

A fluorescence microscopy image showing a complex network of cells. The cells are stained with different fluorescent dyes, creating a vibrant pattern of green, red, and blue against a dark background. The green staining highlights the cell membranes and some internal structures, while the red and blue staining highlights other cellular components. The overall image has a high-contrast, almost abstract quality, resembling a biological web or a microscopic landscape.

# nature medicine

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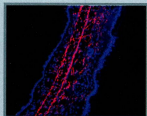
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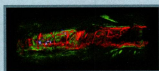
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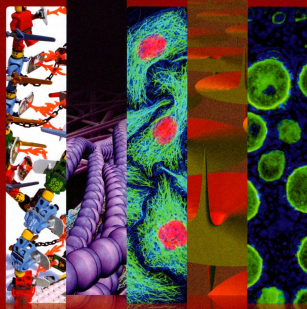
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## The fat of the matter

Amid an obesity epidemic, the approval of two new obesity drugs might revamp the clinical landscape of obesity treatment.

In spite of the growing burden of obesity worldwide, anti-obesity drug development and approval have lagged behind. In the few cases in which a therapy has made the regulatory cut, as with sibutramine and rimonabant, safety concerns have forced regulators to pull the drugs from the market (see *Nat. Med* 18, 843, 2012). These failures over the past 13 years, and in particular the high bar for safety, have dampened the interest of pharmaceutical companies in this therapeutic space. But this situation may change after the approval of two new antiobesity drugs by the US Food and Drug Administration (FDA).

In June, the FDA approved lorcaserin (Belviq), a prescription weight-loss drug developed by Arena Pharmaceuticals that induces satiety through the activation of the 5HT<sub>2C</sub> receptor, a subtype of serotonin receptors found only in the brain. In 2010, the FDA had rejected lorcaserin because of evidence that it increased cancer incidence in rats and because its modest efficacy did not outweigh safety concerns. New data presented by the company changed the views of the FDA advisory panel, which concluded that the cancer findings did not apply to humans.

Obese adults and overweight people with a concomitant weight-related comorbidity (type 2 diabetes, dyslipidemia or hypertension) are eligible for this drug. In two clinical studies carried out with nondiabetic individuals and one follow-up study with subjects with diabetes, lorcaserin led to an average loss of 3% body weight after one year of treatment when adjusted to placebo. Although this is less than the 5% described as clinically significant by the FDA, the studies met other FDA criteria for approving weight-loss drugs—that at least 35% of treated subjects achieve 5% weight loss after one year and that this number is double the number of responders in the placebo group. In individuals with diabetes, lorcaserin also improved glycemic control, regardless of whether the patients lost weight.

The FDA's approval in July of Qsymia, developed by the small drug company Vivus, may signal a greater willingness of the FDA to tolerate certain side effects in an antiobesity drug. Qsymia is a combination of two older drugs, phentermine and topiramate, that reduces hunger by targeting different neurotransmitter systems, mainly norepinephrine but also dopamine and serotonin. Whereas this drug seems to induce more weight loss than lorcaserin in a similar target population (an average of 8.9% at the highest dose compared to placebo), it may cause more serious side effects, including an increased heart rate and increased risk of birth defects. Both Arena and Vivus have committed to

carrying out post-marketing studies to assess adverse events. Given the numerous potential subjects and the required long-term treatment, these studies are crucial, as cardiovascular risk might already be higher in obese people.

Despite lingering concerns regarding potential side effects, the FDA was convinced that the benefits outweigh the risks, as there is evidence that even a 5% weight loss can have positive effects on blood pressure and metabolic syndrome. These drugs may help individuals achieve a consistent degree of weight loss, which may be crucial, as recidivism is common among dieters and a substantial barrier for obesity therapy. At the same time, patient education must clarify the drugs' real efficacy and expected outcome to avoid false expectations. According to the FDA, people should stop taking these drugs if they fail to lose 5% body weight in 12 weeks, something that happened in about 60% of the subjects taking lorcaserin during the clinical studies.

Lorcaserin's approval validates serotonin signaling as an anti-obesity target and suggests that specifically targeting 5HT<sub>2C</sub> receptors could minimize undesired side effects. However, compensatory mechanisms affecting other neurotransmitter systems and circulating hormones that control weight might limit the drug's efficacy. A strategic use of drug combinations may provide synergistic effects by targeting different pathways to increase efficacy or avoid resistance. The development of drug combinations poses a challenge for drug companies and regulatory agencies, yet the complexity of the network regulating energy balance will probably require this multitarget strategy.

The FDA's decision to approve the drug despite its modest efficacy is a reflection of the magnitude of the obesity epidemic. In the US, ~70% of adults are overweight and about 33% and 17% of adults and children, respectively, are obese. Although these numbers are now reaching a plateau in the US, the prevalence remains high, and, globally, obesity rates are predicted to keep on increasing. When considering this huge medical need and the lack of available drugs, it is not surprising that many within the medical community have applauded the approval of these two drugs.

Although Belviq and Qsymia are not perfect drugs, and their use should not replace efforts to encourage long-term weight loss through a healthy lifestyle, they are an important addition to the antiobesity toolbox. These two drugs may be the harbinger of further pharmacological developments to help beat obesity by attacking it through different fronts.

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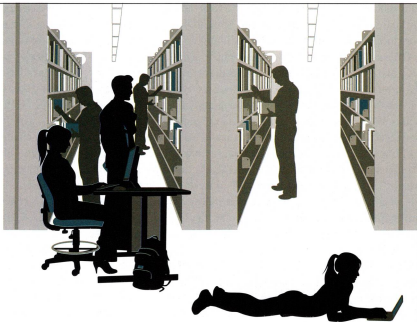
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# natureOUTLOOK

Published in the 17 May issue of *Nature* and available free online for six months

## DIABETES

About 350 million people – 5% of the world's population – are afflicted by either type 1 diabetes, an autoimmune disorder, or type 2 diabetes, largely linked to lifestyle. *Nature Outlook: Diabetes* examines the latest research into the causes, therapy, prevention and impact of these devastating diseases.

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### Getting testy:

Assays developed to predict antipsychotic drug response



### Nothing compares:

The US gets serious about comparative-effectiveness research



### Principal results:

Nurses challenge the idea that only doctors can lead trials

## Point-of-care tests poised to alter course of HIV treatment

Testing for HIV does not simply end with the diagnosis that the virus is present in a patient; caregivers also need to track the disease's progress to adjust ongoing treatment. Yet tests for monitoring HIV infection require sophisticated instruments, well-trained clinicians and expensive lab ware. All those are in short supply on HIV's front line in places such as rural sub-Saharan Africa. "It's a problem not just of cost," explains hematologist Helen Lee of the University of Cambridge in the UK. "It's a problem of having access." In the last year, stripped-down standalone tests have appeared on the market, offering rural patients a cheaper, faster count of their CD4 immune cells. And in the coming months, a class of tests that measure viral load should enter routine point-of-care use, too, offering caregivers a choice of simple tools for measuring HIV infections.

At present, monitoring the virus in patients presents a logistical challenge, even in wealthy areas. In such places, an HIV-positive patient's blood may be drawn every six months by a trained nurse and stored in \$40 worth of reagents, refrigerated and sent to a laboratory where a skilled technician uses a laser-equipped machine costing around \$100,000 to count the CD4 immune cells in the patient's blood. If the count drops below certain thresholds, such as 350 cells per milliliter, implying damage from HIV, the patient's caregivers may initiate antiretroviral therapy or change to a new therapy if an existing one is no longer working. If treatment happens early enough, it can fend off the HIV infection before the patient's immune system is weak enough to allow an opportunistic infection such as tuberculosis. Follow-up tests can help caregivers decide whether the treatment is working and whether to switch to backup drugs.

In resource-poor settings, testing and monitoring the infection might involve a trip to a clinic many miles away. By the time an answer comes back from a far-off laboratory—sometimes as much as a month later—the patient may also be harder to track down and his infection tougher to fight.

The turnaround of results could shorten to hours, rather than weeks, thanks to recent arrivals on the point-of-care market, such as PointCare NOW, the Pima CD4 Analyser



Count it in: The Pima CD4 Analyser.

and the CyFlow™ CD4 miniPOC, which count a patient's CD4 immune system cells in a body fluid such as saliva or blood. These simple CD4 counters could improve survival rates and reduce infection in places with little infrastructure, says immunologist Steven Reid of Imperial College London, who managed a competition to develop a new CD4 point-of-care test. Zyomyx, a Fremont, California-based diagnostics company, won that competition and a round of development funding in 2008 and plans clinical trials of its CD4 test later this year.

Another type of test limited until now to laboratories may soon reach point of care, too. Viral-load tests detect and amplify the nucleic acid present in HIV and give a direct measure of the infection's extent. "CD4 counts drop more slowly than viral load rises," Reid explains, so more researchers favor viral-load testing to monitor the illness. Earlier this year, Lee, through

her spin-off company Diagnostics for the Real World in Sunnyvale, California, conducted a trial in rural Malawi testing a point-of-care HIV test called simple amplification-based assay, or SAMBA. Consistent with what Lee's team previously found with clinical samples in the lab (*J. Infect. Dis.* 201, S65-S72, 2010), the field trial found a 96-98% agreement between the SAMBA test and a standard laboratory assay, prompting the Swiss-based global nonprofit Doctors Without Borders to plan routine implementation of SAMBA in Malawi by the end of this year. Three other viral-load point-of-care tests are already in advanced trials, and a recent report from UNITAID, a UN body, identifies eight more in the pipeline. This type of testing is currently used for monitoring antiretroviral treatment effectiveness, but it might also one day replace CD4 assays for deciding when to begin administering these drugs in the first place, argues Gottfried Hirschall, director of the World Health Organization's HIV/AIDS Department in Geneva.

For now, viral load testing technology is more expensive than CD4 tests. Yet Hirschall, a speaker at the 19th International AIDS Conference in Washington, DC, in July, is optimistic that competition among manufacturers will lower soon drive down those prices. CD4 will serve as a stepping-stone for POC viral load tests, he says. "The new [CD4] tests are competing with the big machines," he says, "ultimately this will be to the benefit of the patients."

Lucas Laursen

#### Select point-of-care HIV assays in the pipeline

| Assay name                              | Company                        | Target measured |
|-----------------------------------------|--------------------------------|-----------------|
| Daktari™ CD4 Counter                    | Daktari Diagnostics            | CD4 cells       |
| MBio™ Diagnostics CD4 System            | MBio Diagnostics               | CD4 cells       |
| CD4 Point of Care Technology            | BD Biosciences                 | CD4 cells       |
| Zyomyx CD4 Test                         | Zyomyx                         | CD4 cells       |
| Semi-quantitative CD4 Test              | Burnet Institute               | CD4 cells       |
| NAT System                              | Alere                          | Viral load      |
| Liat™ Analyser                          | IQuum                          | Viral load      |
| SAMBA                                   | Diagnostics for the Real World | Viral load      |
| EOSCAPE-HIV™ HIV Rapid RNA Assay System | Wave 80 Biosciences            | Viral load      |

Source: HIV/AIDS Diagnostic Technology Landscape, UNITAID (June 2012)



# Race heats up for first-to-market drugs for resistant tuberculosis

In early July, Janssen Research and Development, part of the New Jersey drug giant Johnson & Johnson, announced that it had requested US approval of a new compound called bedaquiline to treat multidrug-resistant tuberculosis (MDR-TB). If approved by the country's Food and Drug Administration (FDA), the medication would be the first tuberculosis drug with a new mechanism of action in four decades, as well as the only drug specifically indicated for MDR-TB, which roughly 650,000 people are living with worldwide, according to the World Health Organization.

The announcement created much fanfare, but Janssen isn't the first company to seek approval for a novel compound to fight MDR-TB. Last December, the Japanese drugmaker Otsuka Pharmaceutical filed for approval of its compound, delamanid, in Europe. With additional incentives signed into law in the US last month, multiple players are vying for the lead in the MDR-TB drug development niche.

At present, treating drug-resistant tuberculosis involves a bevy of regular tuberculosis medicines that, in many cases, must be administered for as long as two years or more. And the drugs don't always work. The hope is that new medicines will shorten treatment times and improve cure rates. Janssen hasn't definitively proven that bedaquiline can do that, but initial results from two phase 2 trials presented at each of the last two Union World Conferences on Lung Health look promising. In the first, a two-year trial of 161 individuals with MDR-TB, participants who received the compound on top of the standard five-drug cocktail for six months experienced a 79% clearance rate of the pathogen from their sputum, compared with 58% in the group that had a placebo added to the regimen. Janssen plans to launch a 600-person phase 3 trial later this year that will examine whether bedaquiline plus an experimental regimen can cut the treatment time for MDR-TB from two years to nine months.

In June, Otsuka published a phase 2 randomized trial of delamanid that included 481 individuals with MDR-TB. Delamanid plus the existing MDR-TB regimen cleared bacteria in nearly half of the participants in the first two months compared with 30% of participants who received the placebo plus standard treatment, the researchers found (*N. Engl. J. Med.* 366, 2151–2160, 2012).

Although Janssen's and Otsuka's MDR-TB

drugs have fared similarly in trials, their mechanisms of action differ markedly. Bedaquiline kills *Mycobacterium tuberculosis* by targeting the key energy enzyme adenosine triphosphate (ATP) synthase, something no other antibiotic does. Delamanid, in contrast, inhibits production of mycolic acid, a molecule found in the bacterium's cell wall.

Which compound will win out if both are approved remains to be seen. Eric Nuermberger, of the Johns Hopkins University School of Medicine's Center for Tuberculosis Research in Baltimore, notes that bedaquiline seems more potent in animal models, but "there's certainly no data from the clinical trials that suggests superiority of one over the other." Notably, bedaquiline has a major drawback: a strong drug-drug interaction with a common first-line drug used to treat tuberculosis. Rifampin speeds up the metabolism of bedaquiline, which means that less of the compound reaches the bacteria.

## Extensive drug development

Researchers are investigating a third compound to treat MDR-TB—Zyvox (linezolid), which is marketed by New York-based Pfizer and has already been approved by FDA to treat other infections such as pneumonia. In a meta-analysis published earlier this year that spanned 12 studies from three continents, researchers found that 99 of 121 patients were successfully treated with Zyvox-containing regimens (*Eur. Respir. J.* doi:10.1183/09031936.00022912, 2012). One disadvantage of Zyvox is that it can have toxic effects involving the bone marrow and the peripheral nervous system, especially when administered for long periods. Other drugs in the same class as Zyvox are currently being developed to treat tuberculosis, including Pfizer's sutezolid, now in phase 2 trials. The hope is that these newer drugs will be safer, says Robert Horsburgh, an epidemiologist at Boston University and chairman of RESIST-TB, an initiative aimed at promoting research into new treatments for drug-resistant tuberculosis.

Other novel compounds are in the pipeline as well. For example, Maryland-based biotech Sequella is developing a tuberculosis treatment called SQ109, which is currently in phase 2 testing. Last year, Maxwell Biotech, a Russian venture capital fund, licensed the rights to develop



Warning heard: MDR-TB patients need drugs.

the compound for MDR-TB in Russia and neighboring countries.

The fact that several companies are seeking approval for drugs to treat MDR-TB as opposed to drug-susceptible tuberculosis is not surprising, says Nuermberger. "It's easier to show treatment benefit, because the treatments that we have for multidrug-resistant TB are vastly inferior to the regimens that we have to treat drug-susceptible disease," he says.

Pharmaceutical companies will soon have another incentive for going after MDR-TB. The fifth reauthorization of the US Prescription Drug User Fee Act (PDUFA), signed into law on 9 July, includes a subsection called the Generating Antibiotic Incentives Now (GAIN) Act that aims to spur development of antibiotics for drug-resistant bacteria, including MDR-TB. Drugmakers that ask for approval of medicines to treat these pathogens will receive priority review, as well as five additional years of market exclusivity and fast-track status. "The goal there is to try to put some more enticements on the table for companies to develop new antimicrobial drugs," says Brad Tebbets, an infectious disease analyst at Global Data, a London-based research firm that tracks the pharmaceutical industry.

Cassandra Willyard

# Potential first-in-class osteoporosis drug speeds through trials

In the case of osteoporosis, a bone disease notoriously difficult to treat, doctors have hailed each new kind of drug as a major therapeutic advance. In 1999, the US Food and Drug Administration approved Merck's bisphosphonate drug Fosamax (alendronate)—which, along with other similar agents, quickly became the standard of care—and, just two years ago, the agency cleared Amgen's targeted RANK ligand inhibitor Prolia (denosumab). In some ways, the drugs have succeeded: Fosamax's sales reached \$3 billion a year before the drug's patent ran out, and Prolia has improved upon the efficacy of earlier drugs. But highly publicized side effects are causing patients to shy away from bisphosphonates, and Prolia, an injectable drug, can be a nuisance to take.

Now, on the heels of these struggles in the osteoporosis market, there's a new potential frontrunner: odanacatib. The oral drug inhibits the bone resorption enzyme cathepsin K and, if approved, will be the first in its class to reach the market. Although Fosamax and Prolia can increase bone mass by shutting down cells known as osteoclasts, they also hinder the creation of new bone. Odanacatib, which comes out of Merck's labs, seems to inhibit bone resorption without any such drawback. "This is a unique drug that may have less of an impact on inhibiting bone formation or, in some cases, actually stimulate bone formation," says Sundee Khosla, an osteoporosis researcher at the Mayo Clinic in Rochester, Minnesota. "This could be something new and different to offer our patients."

Last month, after an outpouring of positive, early data from a pivotal trial on odanacatib, Merck announced that it was taking steps to close the trial early and move along, ahead of schedule, with regulatory applications. The trial, which launched in 2007, included more than 16,000 post-menopausal women over the age of 65. The interim analysis data have not yet been released, but recently published data on odanacatib from primate studies and from a phase 2 human trial (*J. Bone Miner. Res.* doi:10.1002/jbmr.1695, 2012) indicated that, compared with Fosamax, the new drug leads to almost twice as much an increase in bone mineral density in some bones and less reduction in serum markers



Dore Bringer/Photo Researchers, Inc.

**Finding a niche:** New osteoporosis drugs target different pathways that influence bone formation.

of bone formation. Merck, headquartered in Whitehouse Station, New Jersey, plans to file for regulatory approval in the US and the EU early next year.

## A fraction of the fractures

Previous cathepsin K inhibitors from both Novartis and GlaxoSmithKline were halted because of side effects—particularly in the skin, where the enzyme is expressed in addition to bone. Merck has not reported an unusual rate of side effects with odanacatib, but many physicians are reserving judgment until long-term data from ongoing trials are released. "We need to await the results of this exceptionally large trial to make conclusions as far as the overall safety

**"We need to await the results of this exceptionally large trial to make conclusions as far as the overall safety and efficacy is concerned."**

and efficacy is concerned," says Henry Bone, an osteoporosis researcher at the Michigan Bone and Mineral Clinic in Detroit involved in ongoing odanacatib trials for Merck. "But I

think clinicians will be greatly influenced by the actual fracture data [showing a benefit] when they see it."

Clinicians are also keeping a close eye on data from other cathepsin K inhibitors: Japan-based Ono Pharmaceutical's ONO-5334 is in phase 3 trials, and Sweden's Medivir has a drug now in phase 1 testing. Meanwhile, California-based Amgen has an experimental osteoporosis agent that inhibits the protein sclerostin, known to play a part in inhibiting bone formation. The antibody drug, AMG785, which kicked off phase 3 trials earlier this year, is the furthest along in development in its class.

For now, odanacatib is still the experimental osteoporosis drug closest to reaching the market, and if it meets benchmarks on safety and efficacy, market analysts at Credit Suisse in New York project the drug will offer a competitive alternative to bisphosphonates. The drug can be offered as both a first-line osteoporosis treatment and an option for patients who have hit the five-year mark with bisphosphonates and are no longer seeing an effect, the analysts say in a report published 25 June.

*Sarah C P Williams*

## Osteoporosis drugs in ongoing or recently completed phase 3 trials

| Drug        | Lead company       | Target                       |
|-------------|--------------------|------------------------------|
| BAO58       | Radius Health      | Parathyroid hormone receptor |
| Femivia     | EndoCeutics        | Androgen receptors, estrogen |
| Odanacatib  | Merck & Co.        | Cathepsin K                  |
| Ostora      | Tarsa Therapeutics | Calcitonin receptor          |
| Romosozumab | Amgen              | Sclerostin                   |

Source: BioMedTracker

## Overactive bladder, under scrutiny, gets a new treatment

A cop is directing traffic when the urge to urinate suddenly strikes and, overcome, she abandons a busy intersection in search of a restroom. The solution for "always having to go," according to the advertisement in which she appears, is medication for "overactive bladder." In no time, she's back on the job, no longer in any need for those vexing bathroom breaks. In reality, however, many people diagnosed with overactive bladder don't benefit from drugs. And despite the approval in June of a first-of-its-kind drug for the condition, the debate continues as to whether the term itself is a confusing catchall.

Overactive bladder describes a pressing need to urinate due to bladder spasms or contractions, with symptoms that can include repeated trips to the bathroom—even at night—and incontinence. Patients diagnosed with this disorder are typically asked to try behavioral changes, such as decreasing fluid intake and altering diet, as well as exercises to strengthen the pelvic floor muscles to reduce or decrease leakage. If that doesn't work, doctors will prescribe medications that target the symptoms.

Currently, experts estimate that between 30 million to 50 million people in the US alone are diagnosed with 'overactive bladder.' But many experts worry that the term casts too wide a net.

In a recent commentary, Kari Tikkinen, a urologist at McMaster University in Hamilton, Canada, argues that lumping the symptoms together is an oversimplification (*Eur. Urol.* 61,

746–748, 2012). "Calling it one disease, you fail to recognize that there are many reasons for the symptoms, from neurologic dysfunction to prolapsed uterus to prostate disorders that have nothing to do primarily with the bladder," says Tikkinen. "So there's a huge risk that you overtreat people with these drugs. The symptoms should be studied individually to develop effective medications and therapies."

But Alan Wein, head of urology at the University of Pennsylvania Perelman School of Medicine in Philadelphia, who helped coined the term in 1997, defends the diagnosis. "Overactive bladder is a patient-friendly term," he says. "We thought it might get more people that had this condition to be treated."

Antimuscarinics, the current treatment standard, relax the bladder's smooth muscle, reducing involuntary contractions and subsequent leaks. The six antimuscarinics on the market, including the gel Antulol (oxybutynin), from New Jersey-based Antares Pharma, approved last December, all have "reasonable effect," says Roger Dmochowski, a urologist at the Vanderbilt University Medical Center in Nashville, Tennessee. But, he adds, about 30% of people who take them see no response.

### Expanding options

On 28 June, the US Food and Drug Administration approved Astellas Pharma's Myrbetriq (mirabegron), the first in a new class of drugs to treat overactive bladder. The once-

daily pill, a beta-3 adrenergic agonist, relaxes the bladder muscle during filling, improving storage capacity. In phase 3 studies submitted by Astellas to the agency, the drug from the Tokyo-based pharmaceutical company decreased urinations by about 1.7 episodes per day on average from a baseline of about 12, compared with a 1.2-episode reduction with placebo, and cut daily leakage episodes nearly in half, from a baseline of about 3 to 1.5, compared with a reduction of only 1 with placebo. More beta-3 adrenergic agonists are in the pipeline, such as AltheRx Pharmaceuticals' solabegron and Kissei Pharmaceuticals' ribotegron.

Still, some onlookers remain unsuayed by that data. "The studies show these drugs barely work," says Kate Ryan, program coordinator at the National Women's Health Network, a nonprofit advocacy organization in Washington, DC. "These drugs reduce the frequency by less than once per day compared to the placebo, and that is not enough to make a meaningful difference in anyone's life."

Scientists are on the hunt for urine and blood biomarkers for overactive bladder that could refine the diagnosis and lead to more targeted therapies. In the meantime, though, clinicians need to be better about creating reasonable expectations, says Dmochowski. "Patients think that medication is going to cure them," he says. "With current therapies, the majority will be benefited, but not cured."

*Alisa Opar*

## Study finds up to \$5 billion in potential trial cost savings

Extraneous procedures in clinical trials are costing the drug industry up to \$5 billion each year. That's according to a new analysis from the Tufts University Center for the Study of Drug Development, which found that tests not linked to key efficacy or safety trial goals can account for as much as 20% of a clinical study's budget.

According to Ken Getz, who authored the report, if trial sponsors "directly tie the design of their protocols to their primary endpoints so that only useful data is gathered," they could stand to save an average of \$260,000 per phase 2 clinical trial and \$1.7 million per phase 3 study on the direct costs of lab tests or participant questionnaires. For example, in a trial looking at a new obesity medicine, measuring blood sugar could be considered a surplus procedure if only changes in body weight and lipid profiles were defined as the primary and secondary endpoints, respectively.

Getz came to his conclusions after analyzing some 22,000 protocol procedures from a total of 117 mid- and late-stage trials sponsored by 15 different drug companies. He reported the findings of the study, which was funded by the New York-

based software firm Medidata Solutions, on 26 June at the Drug Information Association annual meeting in Philadelphia.

Trimming superfluous procedures within trials could even have added cost-cutting benefits, says Christine Pierre, president of RxTrials, a clinical research organization based in Ellicott City, Maryland. In addition to the direct money saved by not running extra tests, more efficient study designs could help lower expenses related to patient recruitment and retention, she notes. "If we can increase enrollment by streamlining procedures, then we can not only impact patient's willingness to participate, but we might also improve their adherence to the protocol," Pierre says.

But Steven Goodman, an epidemiologist at Stanford University in California and editor of the journal *Clinical Trials*, worries about the consequences of paring trials down too much, noting that extra information can inform future studies or come in handy in case such data is requested by regulatory agencies. "On the surface it may look like certain procedures do not provide necessary measurements, but this data can later be mined," he says.

*Kathleen Raven*

# New tracking system proposed to help recall faulty devices

A miter saw, decorative lights, socks for toddlers—these were a few of the consumer goods recalled off of shelves across the US last month because they posed a danger to users. Recalling faulty pacemakers or catheters is much more difficult, however, because no system exists for tracking medical devices on the market. In an effort to remedy that and boost consumer safety, on 3 July the US Food and Drug Administration (FDA) outlined plans to create a new comprehensive monitoring system for medical devices.

“The FDA took on something very ambitious here,” says Jonathan Gaev, a senior engineer at the ECRI Institute, a nonprofit healthcare research organization based in Plymouth Meeting, Pennsylvania. “It is trying to produce something that addresses a wide range of devices, that is not unduly burdensome and that will be a big step forward for adverse-event reporting.”

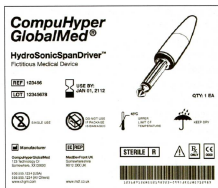
The proposed rule will require most devices to carry a unique device identifier (UDI) tag similar to the barcode found on most consumer products—either on the product label or packaging, or, in cases where the label and the device are likely to be separated such as implantable devices, on the equipment itself. The markings will be phased in gradually, with the riskiest devices to carry the codes within

a year after the rules are finalized, which is expected to happen sometime next year. The FDA will then house those numbers in a publically accessible database. The cost for implementing the system—estimated at more than \$500 million over the next decade—will be largely shouldered by device makers.

The FDA began developing the tracking system about a decade ago, in response to incidents in which healthcare facilities that received recall notices were unable to tell which device models they referred to because these products lacked a singular identifier. Congress then charged the agency with devising labeling requirements through the 2007 FDA Amendments Act, but that bill set no firm deadlines on implementation. Frustrated by the lack of progress, lawmakers included a provision in this year's FDA Safety and Innovation Act, which also reauthorized and updated the Prescription Drug User Fee Act (see *Nat. Med.* 18, 990, 2012), that compelled the agency to release draft regulations for monitoring devices by the end of this year.

## Slow and steady wins the race

According to Jay Crowley, senior advisor for patient safety at the FDA's Center for Devices and Radiological Health, the agency needed all that time to determine which devices should be



**Marked change:** An example of a proposed UDI.

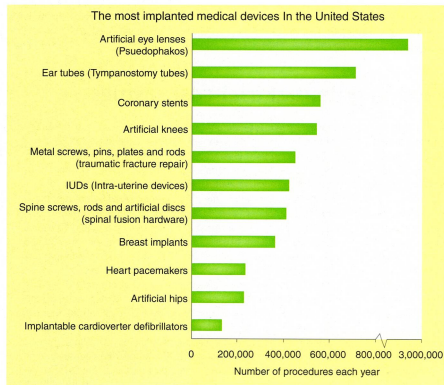
tagged with UDIs and to harmonize the system with parallel efforts planned or in progress in other countries around the world. “We really wanted to be thoughtful about how we approached this, recognizing it's a significant cost and burden for industry, and we wanted the benefits to be tangible for all stakeholders,” he says.

But the devil is in the details, Gaev notes, and a few will probably need refining. For example, the draft rule doesn't require UDIs for simple devices, such as bedpans used in hospitals, or any device sold over the counter. People generally agree that it makes sense to excuse basic, low-risk devices. However, Gaev worries that the exemption for over-the-counter devices might ultimately prove too broad and fail to cover more complex devices for home use developed in the future.

Consumer advocacy groups and device manufacturers broadly welcome the initiative but say they plan to study the details and submit comments on the proposed rules. In a statement, Janet Trunzo, vice president for technology and regulatory affairs at the Advanced Medical Technology Association, a Washington, DC-based trade group, said, “We will be paying particular attention to whether the proposed rule follows a risk-based and least burdensome approach to implementing the UDI system.”

Among its many provisions, the FDA Safety and Innovation Act, which President Barack Obama signed into law on 9 July, originally included a plan for a national ‘track and trace’ system for prescription drugs called RxTEC. However, that proposal was dropped from the final bill because legislators couldn't agree over details such as who would pay for the scheme. California plans to institute a yet more stringent track-and-trace measure in 2015; other US states and various countries around the world also intend to establish such a system.

Alla Katsnelson



Source: /the US National Health Interview Survey



# Clinical efficacy data on gene tests trails marketing in psychiatry

For psychiatric patients today, finding the right drug at the right dose is almost always a matter of trial and error. In recent months, companies have come forward with what they say is a solution to this problem: new commercial genetic tests that purport to predict optimal drug dosing and help avoid adverse events. The arrival of these tests on the market has ignited a debate, however, as many psychiatrists worry that evidence demonstrating their benefit is lacking.

"There's not enough prospective data with most of these genes to as yet guide clinical practice," says Anil Malhotra, director of the Laboratory of Molecular Psychiatry at the Zucker Hillside Hospital, a psychiatric facility in Glen Oaks, New York.

That doesn't mean that researchers and companies aren't trying to fill the data gap. One of the first such prospective, randomized studies was reported in late May in Phoenix at an annual psychopharmacologic conference known as the New Clinical Drug Evaluation Unit meeting. A team led by Kevin Fumaga at the Pine Rest Christian Mental Health Services in Grand Rapids, Michigan, randomly assigned 51 people with depression to receive either a test called GeneSightRx Psychotropic, which tests for a handful of DNA polymorphisms linked to drug metabolism and response, or treatment as usual. All participants swabbed their cheeks for DNA but subsequently didn't know whether their care was guided by pharmacogenomics. Clinicians who scored the patients' outcome measures were also blinded to which study subjects received the pharmacogenomic test.

Although the trial proved too small to yield statistically significant results, the majority of the 26 participating psychiatrists used the test to either choose or confirm their primary treatment. Furthermore, the pharmacogenetically guided therapy yielded a trend toward improved clinical outcomes, with half the subjects receiving the test responding to their therapy compared to only a quarter in the treatment-as-usual arm.

Currently, the US Food and Drug Administration requires drug labels to contain information about pharmacogenetic biomarkers for 26 different psychiatric medications, including many best-selling antidepressants and antipsychotics such as Celexa (citalopram) and Risperdal (risperidone). These label warnings are based on many studies that have demonstrated correlations between certain mutations in certain liver-detoxifying enzymes and drug



How do you do: Genes up Celexa response.

responses. Yet, few of these labels offer much in the way of clinical recommendations for how to use this information.

Diagnostics companies are forging ahead with a fix. On 28 June, California-based Millennium Laboratories started marketing a test to physicians that analyzes variations in genes encoding enzymes associated with the metabolism of drugs commonly prescribed to people suffering from pain, anxiety, depression or insomnia. The month before, AssureRx Health rolled out GeneSightRx ADHD, which measures variations in three genes linked with response to medications routinely used to treat attention deficit hyperactivity disorder. It is the second product from the Ohio company, which began selling the GeneSightRx Psychotropic test in 2010.

According to AssureRx's president and chief executive James Burns, both GeneSightRx tests "are easy; they're actionable and they're immediately applicable in a clinical setting."

## Predictive guidance

Many psychiatrists welcome these new tests—some to guide drug choice, but the majority for dosing decisions. "I think we know a lot more about trying to prevent side effects than good drug response," says David Mrazek of the Mayo Clinic in Rochester, Minnesota, who wrote the 2010 textbook *Psychiatric Pharmacogenomics* and sits on AssureRx's scientific advisory board. "Although it would be great to tell patients that this drug will work for them, we're not there yet."

Given the preliminary state of the science, Roy Perlis, director of the Psychiatry Center for Experimental Drugs and Diagnostics at the Massachusetts General Hospital in Boston, thinks the tests should only be rolled out for a subset of mental health patients. "It would absolutely be a mistake at this point to get these tests in everyone," Perlis says. "My suspicion is that these kinds of tests may be more useful for

patients who have failed multiple treatments and not gotten better than they are for all comers."

That remains a hypothesis at this point, but it's a testable one—and most companies in the field are now actively engaged in clinical research to assess how best to market their products. Last year, for example, AssureRx, together with scientists at the New Jersey-based Medco Research Institute, launched a 2,000-person study to examine whether drug adherence rates are higher and medical claims less frequent for people who receive the GeneSightRx Psychotropic test, all of whom must have recently switched or added antidepressant and antipsychotic drugs, compared to matched controls from Medco's databases.

Genomind, the Pennsylvania-based startup behind a ten-gene psychiatric pharmacogenetic test called Genecept, which has been commercially available for about a year now, is similarly sponsoring a handful of both open-label and blinded studies to assess the company's assay in people with tough-to-treat depression. "We take the science very seriously," says Nancy Grden, general manager of Genomind, which counts both Perlis and Malhotra on its scientific advisory board. "We know [the test] is not the be-all and end-all, but it's certainly better than the trial and error that's going on today, and we think it is important to have as much data as possible to share for the whole discipline."

The issue of cost also remains unresolved at this point. In one of the only published models to date, Perlis and his colleagues estimated the sticker price of using pharmacogenomic testing to decide between two classes of antidepressants relative to the next best strategy of simply prescribing one type of drug first and then switching sequentially if treatments don't work. Assuming a test costs \$500, which is in the right ballpark for most of today's products, Perlis's team calculated that, for a 40-year-old with major depression, each quality-adjusted life year gained because of pharmacogenomic testing would cost close to \$100,000, which is on the border of what economists say is a cost-effective measure (*Neuropsychopharmacology* 34, 2227–2236, 2009).

"There are a lot of questions about the use of genetic testing in psychiatry," notes Fumaga. "But this is the direction that the research has to take if [psychiatric pharmacogenetic testing] is going to be a legitimate technology with credibility."

Elie Dolgin

# Biomedical briefing

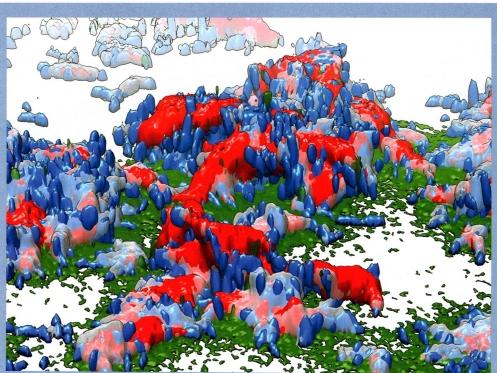
## POLICY

### Border traffic

Drug companies and researchers wishing to conduct cross-border clinical trials in Europe might no longer have to submit separate applications in each country where they hope to conduct part of a single study. On 17 July, the European Commission introduced draft rules that would allow a centralized application process for all EU member states. In a press conference, Health Commissioner John Dalli said the reforms are intended to maintain "a high level of patient safety and rights while reducing the administrative hurdles for clinical excellence in Europe." The proposals must now be ratified by Europe's Parliament and Council of Ministers before becoming law.

### Free medicines

The Indian government has started to set aside money for an ambitious \$5.4 billion, five-year plan to provide the country's 1.2 billion inhabitants with hundreds of different types of generic drugs for free. The health ministry estimates that, as a result of the new program, which is set to launch in October, the percentage of Indians receiving public healthcare will increase from the current 22% to 52% within five years. K.M. Venkat Narayan, a policy researcher at the Emory University Rollins School of Public Health in Atlanta, applauds the government effort but argues that more is needed to boost the state of healthcare in India. "The Indian health system needs strengthening to address doctor and nurse shortages and a lack of quality assurance and accountability measures," he says.



### Bacterial plaque-forming targets caught on biofilm

Biofilms are not the prettiest things to look at. But by watching these slimy bacterial surface coverings form with nanometer precision, researchers have revealed the architectural roles of four key components that could be targeted to break up biofilms and expose the bacterial community within them to the killing power of antibiotics. With the help of a new microbial labeling strategy, a team led by biophysicist VeySEL Berk at the University of California–Berkeley showed that the Bap1 protein (pictured

here in green) helps cholera cells (blue) attach to surfaces. The cells stick together with the help of the RbmA protein (gray), whereas a protective shell (red) made of the RbmC protein and a polysaccharide sugar envelops the bacterial clusters (*Science* **337**, 236–239, 2012). "With this technology right now, we know what kind of proteins [the bacteria] are using to build their houses," Berk says, "and we can use that information to develop much smarter ways of treating diseases."

### Collaboration cure

At last month's International AIDS Conference held in Washington, DC, a working group assembled by the Geneva-based International AIDS Society presented seven recommendations to accelerate HIV cure research, including the development of new assays to detect latently infected cells (*Nat. Rev. Immunol.* doi:10.1038/nri3262, 2012). "We think the recommendations will become an effective road-

map for the global community interested in HIV research," says working group co-chair Steven Deeks of the University of California–San Francisco, who also coauthored a call for a calling for a consortium dedicated to cure research supported by an annual multimillion-dollar budget (*Nature*, **487**, 293–294, 2012). In the US, only 2% of the National Institutes of Health's (NIH's) \$3.1 billion budget for HIV/AIDS research in 2011 went toward cure studies.

## PEOPLE

### Wellcome wisdom

Mark Walport, the director of the Wellcome Trust, has been tapped as the UK's next chief scientific adviser. "He's a really good fit," says Beck Smith, assistant director of the Campaign for Science & Engineering, an advocacy group based in London. "He's a really good champion for science, and I think that's exactly what that role should be." Walport, a clinical immunologist

who has led the Wellcome Trust for nearly a decade, will replace population biologist John Beddington in the influential role in April 2013. The London-based charity, the largest non-governmental funder of medical research after the Bill & Melinda Gates Foundation, is now looking for a new director.

## A born scholar

The NIH has named its first appointee to the Lasker Clinical Research Scholar Program, an elite training initiative that aims to breed the next generation of physician-scientists. On 3 July, cardiologist Nehal Mehta (pictured) moved his laboratory from the University of Pennsylvania Perelman School of Medicine in Philadelphia to the National Heart, Lung and Blood Institute in Bethesda, Maryland, where he will have five to seven years of funding as head of the new Laboratory of Inflammation and Cardiometabolic Diseases. "Because of the resources I am now afforded, I have doubled the size of my team. I have doubled the size of my lab and I have probably near quadrupled the size of my national recruitment program [of patient samples]," Mehta says. After his initial stint, Mehta will then have the



University of Pennsylvania Perelman School of Medicine

choice to stay on at the NIH as a tenured investigator or to join an academic institution with up to five more years of agency support.

## BUSINESS

### Fraud settlement

The British pharma giant GlaxoSmithKline agreed last month to plead guilty to criminal charges in the US and pay \$3 billion to federal and state governments for promoting antidepressants for unapproved uses and for withholding safety data about a best-selling diabetes drug. The fine over the illegal marketing of Paxil (paroxetine), Wellbutrin (bupropion), Avandia (rosiglitazone) and a half-dozen other drugs was

the largest such settlement in US history. "These really large settlements may be having an impact," says Aaron Kesselheim, a physician and health policy researcher at the Brigham and Women's Hospital in Boston. "If you listen to the pharmaceutical companies, they'll tell you that this behavior was rife in the industry in the past and they've changed their ways."

### Fat fighters

Obesity in the US is expected to rise from 36% today to 42% by 2030, but a pair of newly approved diet drugs could help reverse that trend. On 27 June, San Diego-based Arena Pharmaceuticals won the go-ahead for its serotonin receptor activator Belviq (lorcaserin), the first new weight loss pill approved the US Food and Drug Administration (FDA) in 13 years. Three weeks later, the agency also cleared a combination of an appetite suppressant called phentermine and an anticonvulsant known as topiramate, which are marketed together as Qsymia by Vivus of Mountain View, California.

### Amylin amassed

The New York-based pharma giant Bristol-Myers Squibb

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[go.nature.com/fg2ip1](http://go.nature.com/fg2ip1)

Compensation demanded for injured subjects:

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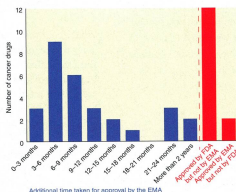
Participant-driven health research on the rise:

[go.nature.com/31RVyl](http://go.nature.com/31RVyl)

(BMS) agreed in late June to pay \$5.3 billion to acquire Amylin Pharmaceuticals and said it would assume the San Diego-based diabetes specialist's debt, bringing the total transaction to \$7 billion. With Amylin's Bydureon—a newly approved, once-weekly version of the company's twice-daily diabetes drug Byetta (exenatide)—BMS should be well positioned to compete against Novo Nordisk and Eli Lilly, the respective makers of the bestselling diabetes agents Victoza (liraglutide) and Lantus (insulin glargine). "BMS will be fighting a two-front battle with those companies," says Michael King, a biotech analyst at Rodman & Renshaw in New York. AstraZeneca, headquartered in London, will pay BMS \$3.4 billion to help co-develop Amylin's pipeline.

## Greater drug access (and greater costs) for US cancer patients

Patients with cancer in the US get faster access to the latest oncology drugs than their counterparts in Europe, but they generally pay more for the privilege. According to a study published last month by Josh Cohen at the Tufts University Center for the Study of Drug Development in Boston, the FDA approved 33% more innovative cancer medicines over the past decade than the European Medicines Agency (EMA), and, in cases when both agencies gave the green light, the FDA was always first to do so (*Tufts CSDD Impact Rep.* 14 (4), 1–6, 2012). In the US, however, oncology drugs are priced on average 9% higher than they are in Europe. Plus, US patients often incur out-of-pocket costs running into the thousands of dollars per year, whereas Europeans, depending on where they live, typically pay only a nominal prescription-filling fee or nothing at all. "It's a tradeoff," says Cohen. "While patients in Europe don't have as many options available to them, the options they do have are basically free."



## RESEARCH

### Diagnostic dividend

The prognosis looks good for the NIH's Undiagnosed Diseases Program. Last month, the agency announced plans to expand the four-year-old program with \$145 million in support over the next seven years. With a new network of academic medical centers across the country and training fellowships in modern genomic analyses, "there will be more breadth in terms of expertise," says program director William Gahl, clinical head of the National Human Genome Research Institute in Bethesda, Maryland.



## Straight talk with...

### Joe Selby

The US Supreme Court's ruling in late June to uphold most of the Patient Protection and Affordable Care Act of 2010 decided more than just the fate of the law's individual mandate for health care. It also allowed many of the other provisions of the legislation to safely go ahead. Chief among those was the establishment of a comparative-effectiveness research hub called the Patient-Centered Outcomes Research Institute (PCORI), located in Washington, DC. With a budget of \$3.3 billion over the next seven years and an independent status, PCORI is now tasked with creating the evidence base to help patients and doctors make more informed decisions about their medical choices.

Leading the fledgling institute is executive director Joe Selby. A family physician and clinical epidemiologist, Selby joined PCORI in July 2011 after 13 years as head of research at Kaiser Permanente, the California-based health provider. Even amid all the uncertainty that surrounded the future of US healthcare reform, Selby, together with PCORI's board of directors, managed to help craft the institute's first research agenda and to fund 50 pilot projects totaling \$30 million, all in the spring of this year. **Elie Dolgin** spoke with Selby to discuss how PCORI will influence the debate about which treatments work best.

#### What was your reaction to the Supreme Court decision?

It was a great relief. The biggest fear is that we would continue living under a cloud of uncertainty. It's been difficult to make progress, particularly with recruiting scientists and other staff. So, with that decision and its definitive answer to the question of whether the Affordable Care Act stands, we've been able to really gear up the hiring process and move forward in other areas.

#### How will PCORI be patient centered, as the name suggests?

In all of our funding announcements we say that you must have patients on the research team working with the investigators to make sure that

the questions are right, the outcomes are right and that at the end that you report the findings accurately and you disseminate the findings broadly. In addition, around 15%, or approximately 3 people on each committee of 20 reviewers, will be patients. That's much more in the way of patient involvement in either the research or the review process than you've seen before.

#### Can you briefly describe the research agenda you adopted in May?

There are five big areas the agenda is designed to address. The first one is the assessment of prevention, diagnosis and treatment options. That, you might say, is classic comparative-effectiveness research. We are putting more of our funding—about 40%—into that priority than any of the others. The second priority is about improving healthcare systems—all of those kinds of system-level interventions that we think really play a big role in whether the basic comparative-effectiveness research gets implemented. The third one is communication and dissemination research. The fourth priority is addressing health disparities, which involves looking at patient subgroups to find out whether treatments work the same in every group, and [why] sometimes they don't. And the last one is called accelerating patient-centered outcomes research. That is about building an infrastructure so that this important kind of research can be efficiently and repeatedly run using data from real-world practice settings.

#### Many of the first pilot projects announced in June seem to be looking at communication strategies or decision-support tools.

That was by design. If it was a comparative-effectiveness research question for the pilot projects, we rejected it. And the reason we did that is because the board felt, and I think they were right, that we could not go funding comparative-effectiveness research until we had done the research agenda, and doing the research agenda took time. So, in the meanwhile, we did these pilot projects.

#### How will PCORI work with the other major US funders of biomedical research, the National Institutes of Health (NIH) and the Agency for Healthcare Research and Quality (AHRQ)?

We have the directors of each agency as members of our board and members of our program development committee, which is a committee of the board that makes decisions about our funding announcements. So, we have them at the table every time that we're considering a funding announcement, and that helps makes certain that we coordinate with those agencies so that our funding is not redundant.

#### What about the Food and Drug Administration?

They're a regulatory entity, and we're anything but a regulatory entity. But I think that there are ways in which we can work together. They can be a source of ideas for us for questions that need to be studied, and there are a lot of methodological questions that we share in common about better ways to evaluate treatment alternatives, both prior to approval and after approval.

#### Will PCORI fund cost-effectiveness research?

No. We're very clear in our funding announcements that we do not fund—in fact, we don't even review—proposals that have cost-effectiveness analyses in them or projects that propose to compare the cost of care for two different treatments. It has to do with the intent of the legislation, both as written and as described to us by people who were there [in Congress]. We feel that we have our plates full with the comparative clinical-effectiveness research, and, in many ways, if you're going to do a cost-effectiveness analysis, you need good comparative clinical-outcomes data first. That's our business.



# Nurses on trial



Rebecca Hersher/UC Irvine Communications

When it comes to clinical trials, physicians almost always run the show. But a determined group of nurses is taking the reins, which some hope will lead to improved patient recruitment, particularly in women's health. **Rebecca Hersher** looks into whether the nurse is the principal investigator of the future.

The air in the ground-floor offices of the memory disorders clinic at Georgetown University in Washington, DC has the warm, close smell of printer ink. Dozens of five-inch binders line the walls, each one filled with paper lists of patients and doctors, research protocol identification numbers and drug manufacturers. They sport titles such as *IRB Correspondence and Study Reference Manual*, along with the names of sponsors: Pfizer, Janssen, the National Institute on Aging. A crowded shelf closest to the main conference table sags under the weight of all the paper.

Although little space remains to add to the collection, the clinic team is busy assembling a new set of binders. Georgetown is one of 26 sites in the US enrolling participants in a phase 2 clinical trial underway to test whether the red wine derivative resveratrol slows memory loss in people with Alzheimer's disease. Before a single subject could sign up for the trial, a study protocol had to be approved by the US Food and Drug Administration (FDA),

consent forms had to be created, and patient-care flowcharts had to be drawn up. A small army of people had to be hired to assist in the trial, among them a study coordinator, a data analyst, a consulting neurologist, two research assistants and a cadre of nurses.

As with every clinical trial, one leader known as the principal investigator is in charge of commanding that army and is responsible for every aspect of the study, from patient care to paperwork. The person in that position for the resveratrol trial at Georgetown is Brigid Reynolds. She has been working on clinical trials for nearly a decade, and this is the ninth time she has been appointed to lead a study. "I love being a principal investigator," she explains. "I get to wear many hats."

But Reynolds is an anomaly among principal investigators, and the letters 'NP' stitched after her name above the breast pocket of her starched white clinic jacket explain why. Reynolds is a nurse practitioner,

a rarity for a person in her position. "Some [physicians] are still surprised when they see a nurse practitioner doing this work," she says diplomatically. "They want to see proof that I'm qualified."

Reynolds is part of an increasingly established group of nurses who lead trials that range from testing the safety of new cancer drugs to evaluating the effect of novel psychotherapies. Because nurses have more time in their practices to ask patients about how lifestyle factors such as sleep patterns, work-related stresses or family relationships play into pathology, they say that the studies they lead yield more comprehensive portraits of potential treatments. Clinical trial ethicists also point out that nurses might help recruit more women to enroll in studies because the nurses themselves are primarily women, and patients gravitate toward trials led by people of their gender. But despite the fact that more nurses hope to supervise trials than ever before, they face an uphill battle to convince sponsors



PH: HUMPHREY/GETTY IMAGES

**Principals with principle:** Brigid Reynolds (pictured above) and Ruth Mulnard (previous page).

who still overwhelmingly choose doctors to fill such positions, in part because physicians possess the power to prescribe any medication.

### Reigning over the trial

The first principal investigator was arguably King Nebuchadnezzar of Babylon. According to the Old Testament, he noticed that his Semitic prisoners, who ate the Jewish diet of beans, lentils and water, were healthier than their Babylonian counterparts who consumed a diet of wine and pork. Nebuchadnezzar took the observational results to heart and switched all of his subjects to the Jewish diet.

The modern principal investigator, however, did not exist until the mid-twentieth century—2,500 years after Nebuchadnezzar—when most countries revised their drug trial regulations to include more stringent guidelines about everything from how protocols are written down to who can examine study participants in the clinic to the minutiae of proper record-keeping. Although regulations in Europe and the US do not specifically require that physicians run studies, “there is a general sense that MDs can be trusted most,” says Ken Getz, a senior research fellow at the Tufts Center for the Study of Drug Development in Boston.

As a result, at least 95% of principal investigators today are MDs, and the remaining 5% are mostly osteopaths and scientists holding doctoral degrees, according to unpublished data collected by Getz and his colleagues. “It’s probably not more than 1% of investigators who are nurses,” he says, “and even that is an increase from 20 years ago when there were virtually none.”

For most of the twentieth century, few nurses earned more than the master’s degree necessary for their profession, fueling the stereotype that doctors are uniquely qualified to lead trials. But a 2011 report by the US Institute of Medicine found that the number of nurses with doctoral degrees in nursing has more than doubled in the last two decades, from 6.5% of registered nurses in 1988 to 13.2% in 2008 (ref. 1). Such degrees prepare nurses to lead clinical trials by giving them firsthand experience with research and teaching them about protocol designs. For example, the University of Washington School of Nursing in Seattle, which *US News and World Report* has ranked number 1 in the country since 1984, requires those seeking nursing science PhDs to take courses on research design and clinical methodology.

Some physicians may actually be less equipped for clinical trial leadership straight out of school than nurses. “Right now, there’s no required medical curriculum for clinical trial training,” explains Ann Bonham, chief scientific officer for the Association of American Medical Colleges, based in Washington, DC. “We don’t have a cogent strategy.”

Ruth Mulnard, who holds a PhD in nursing and is a founding faculty member of the nursing science program at the University of California–Irvine, says she obtained her doctoral degree specifically with clinical trial leadership in mind. It paid off: since 1991

she has run more than 30 clinical trials, the majority of which tested potential new drugs for dementia. Her latest was a 1,000-person, phase 3 trial of solanezumab, an Alzheimer’s drug from Indiana-based Eli Lilly. The study wrapped up in June, and the data from it is currently being analyzed.

### A whole new approach

Many nurses argue that principal investigators like Mulnard or Reynolds bring better “whole patient” care to trials than doctors do. “Nurses have more training in addressing both medical and behavioral aspects of a person’s care,” explains Reynolds. Patricia Grady, director of the National Institute of Nursing Research (NINR), one of the 27 branches of the US National Institutes of Health in Bethesda, Maryland, says nurses ask questions that doctors often don’t think of.

“Often on drug trials, for example, nurses will add on questions about quality of life,” she explains. Such questions help determine whether, if a medication is approved, people prescribed it will adhere to their doctor’s orders or avoid taking the drug because, for example, it disrupts their sleep or makes them thirsty. “The FDA is increasingly looking for such [quality of life] questions on protocols, and those are the kinds of questions nurses are trained to ask,” says Grady.

At Georgetown, Reynolds says her whole-patient approach even helps keep study enrollment up. She typically calls prospective participants and their families over the phone

so that “by the time someone comes in for a screening visit [to be enrolled in the trial], they know what’s going on and they are not apprehensive or confused.” Her attention to the emotions of patients pays off: Reynolds has met the enrollment target in every

one of her seven previous trials in people with Alzheimer’s disease. Among those studies were two early-stage studies of Pfizer’s bapineuzumab, an amyloid-beta-specific antibody that, like Lilly’s solanezumab, is now in phase 3 testing.

In addition to improving protocol design and addressing patient behavior, hiring more nurses as principal investigators could also help recruit more women to participate in clinical trials. A 2007 Tufts study found that 89% of principal investigators were male, and co-author Getz warns that, “in the next ten years, if more women aren’t leading trials, gender disparities are going to get very pronounced.”

Getz says there is mounting evidence that

“In general  
someone with an  
RN just does not  
qualify.”

the gender of the principal investigator affects who chooses to enroll in a clinical trial. "A male doctor talking to a male patient [about a clinical trial] is going to resonate more," notes Christine Carter, vice president of scientific affairs at the Society for Women's Health Research, an advocacy organization based in Washington, DC. Because over 90% of US nurses are female, increasing the proportion of trials run by health professionals with nurse training could help ease the impending gender disparity in clinical trial enrollment.

Across the country at the University of Washington, Pamela Mitchell has also found her training as a nurse valuable in her role as principal investigator. Mitchell has designed and led dozens of clinical studies over her career, nearly all of them testing behavioral endpoints. Behavioral studies make up the vast majority of nurse-led research funded by the NINR, the agency reports. "Nurses operate at the interface between biology and behavior," says Grady, "and especially with an aging population and more chronic disease, developing better patient care is as important as discovering new drugs." One of Mitchell's largest studies found that adding talk-therapy to standard antidepressant drug treatment lessened depression in people who had suffered strokes. In the 100-person phase 3 study published in the journal *Stroke* in 2009, Mitchell found only 27% of people who received talk therapy reported symptoms of depression one year later, whereas 48% of

people on antidepressants alone continued to struggle with the condition<sup>3</sup>.

### Seeking respect

Despite the benefits for patients and a growing track record of success, there is very little demand for nurse principal investigators by study sponsors, such as pharmaceutical companies, that do the hiring. Often a sponsor will hire a third party known as a contract research organization, or CRO, to recommend principal investigators and supervise the trial. And sponsors and CRO players alike share an intense aversion to risk. "Conducting clinical trials is so risky and expensive that the people paying want to make absolutely sure that the

most credentialed people are in charge," Getz explains.

Although Eli Lilly has sponsored studies run by both Reynolds and Mulnard, the company insists that such appointments remain rare exceptions. Lilly spokesperson Eva Groves points out that even in those nurse-led studies, the vast majority of the 20 or more sites enrolling patients had MDs in charge. Covance, a New Jersey-based CRO, hires only MDs as principal investigators, according to senior project manager Brenda Reese, and Quintiles, the largest CRO in the world, says nearly all of its approximately 84,000 North American principal investigators are doctors. Quintiles vice president for early-stage research Oren Cohen says the company favors MDs for leadership positions because

federal regulations require the leader of a trial to be capable of assuming full medical care of study subjects. "Most nurses can't prescribe medication," he points out, so, "in general someone with an RN just does not qualify."

But nurses contend that such hiring practices actually lack a foundation in federal regulation and are downright discriminatory. FDA regulations do not require that the principal investigator be the one to prescribe medication, only that he or she supervise the overall care of study subjects. "The FDA is far more concerned with the experience qualifications of the team than it is with the prescribing ability of the principal investigator," says Mulnard. FDA spokeswoman Michelle Bolek confirms that the agency has no problem with nurses leading clinical trials. "Any medical professional who can fulfill the investigator responsibilities for a particular study is eligible to be a principal investigator," she wrote in an email.

Still, even after leading more than 30 successful trials, Mulnard says there are sponsors who won't hire her because she is a nurse. She says she has had to fight to be taken seriously, and that even now, after all her experience and success, she still hears grumbling from doctors who don't like being supervised by a nurse. "There's always a little undercurrent of threat," she says.

Back at Georgetown, Reynolds says the lack of a true meritocracy in hiring principal investigators has a detrimental effect on the quality of clinical trials. "I've seen less qualified doctors chosen over very experienced nurses," she says.

As she continues to oversee preparations for the upcoming resveratrol trial, however, no such worries are on her mind. Reynolds is entirely in control—meeting with her study coordinator to discuss recruitment, consulting with a neuroradiologist about reading patient brain images and sitting down with sponsor representatives to discuss protocol minutiae. Such acceptance of the nurse as principal investigator is what she and others hope will become mainstream. "There is growing acceptance of the value of the nurse as clinical trial leader," she says. "But we're still working to convince people."

**Rebecca Hersher, a former news intern at Nature Medicine, is currently a production assistant at National Public Radio in Washington, DC.**

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"I've seen less qualified doctors chosen over very experienced nurses."



Chris McLean/US Medicine

**Woman at the top:** Nurses like Pamela Mitchell (center) could attract female subjects to trials.

# The time for pharmaceutical compulsory licensing has expired

Randall Kuhn & Reed F Beall

**The compulsory license mechanism is broken and will not bring drug access to the world's poorest nations. It's time to consider another option—a tax levied on patents—to fund drugs for developing countries, rather than the erratic compulsory licensing mechanism.**



Randall Kuhn/Reed Beall

This past spring, China's State Intellectual Property Office sent a concerning message to pharmaceutical companies around the globe when it announced a revision to the country's patent law that would make it easier to issue compulsory licenses, which are government-issued permits that allow for the targeted production of medicines without the consent of the patent title holder. This move comes 16 years after the World Trade Organization (WTO) in Geneva upheld the right of member states to issue such licenses. But the scale of China's market power clearly has many people paying close attention to the country's next move—all the more because the law allows for drugs made through compulsory licenses to be sold abroad. One can even imagine a day in the future when China might usurp India's leading role in bringing low-cost essential medicines to the world's poorest nations.

Unfortunately, compulsory licenses have historically proven to be an unwieldy tool for promoting pharmaceutical access, especially in poor countries. Our recent evaluation uncovered only 24 episodes since 1995 in which a government official even proposed a compulsory license, with substantially reduced activity since 2008 (*PLoS Med.* 9, e1001154, 2012). This is in part because patent holders—even though they carry no formal status at the WTO—wield tremendous force by threatening to withdraw their products from the local market or by encouraging their powerful host nations to retaliate against weaker nations.

Pharmaceutical firms are well armed and motivated to fight any potential threat to the existing system. Producers will gladly pay a steep one-time charge to buy off any licensing threats and protect the golden goose. They are helped by the fact that health ministries are generally more interested in receiving price reductions for specific drugs than in dramatically overhauling the patent system. More than half of the episodes in our analysis in which a government official initiated a compulsory licensing process ended with the producer issuing a voluntary license, giving a discount, or simply giving the branded drugs away.

Compulsory licensing activity has been especially rare in low-income countries that were purported beneficiaries of the Doha Declaration, an agreement in 2001 by WTO members. Doha specifically sought to address the fact that many poor nations lack the capacity to produce generic pharmaceuticals by allowing producer nations to export medicines to low-capacity countries under a compulsory license. But we found just one such use of this mechanism. In 2007, Canada exported the HIV/AIDS drug Apo-TriAvis (lamivudine/nevirapine/zidovudine) to Rwanda, a resource- and time-intensive fiasco that did little more than discourage other nations from following suit.

The threat of compulsory licenses did play a part in forcing steep discounts or donations of drugs that treat HIV. For instance, beginning in 2006 Gilead Sciences of Foster City, California, established voluntary licensing and distribution partnerships with Indian generics producers, UNITAID and the Clinton Health Access Initiative to distribute a generic version of tenofovir (brand name Viread) at reduced cost in

low-income countries, with a current price of \$159 per year in 70 such countries compared to about \$7,000 in rich countries. Notably, the list excluded China. Now that China has made its move, Gilead is reportedly negotiating to donate a substantial amount of tenofovir to China.

Pharmaceutical manufacturers surely must perceive a danger to the patent system that underpins their business model. That's in no small part because China's announcement came hot on the heels of India's decision in March to license Hyderabad-based Natco Pharmaceuticals to produce the cancer treatment Nexavar (sorafenib) over the protests of the patent holder, German drug giant Bayer. Compulsory licenses increasingly target costly cancer treatments in middle-income countries, rather than drugs for communicable diseases in the poorest nations. If countries such as India threaten with more compulsory licenses for drugs that treat noncommunicable diseases, we may see more discounts and donations flow to middle-income countries, possibly at the expense of donations to the poorest nations.

Communicable diseases pose a huge threat to the health of developing nations and cannot be ignored. And although compulsory licensing can act as a failsafe that provides protection in emergencies such as fast-moving epidemics, it is poorly suited to promoting drug access or health equity to address ailments such as malaria and tuberculosis.

Given this reality, we propose an alternative that would more aggressively promote drug access while stabilizing the patent system. With a truly globalized pharmaceutical market size of close to \$1 trillion, even a relatively small intellectual property tax levied on patents annually on the basis of their value could support novel drug development, purchasing and distribution at a level comparable to all of today's public-private

partnerships combined. A progressive rate structure would discourage windfall profit taking. Tax credits or exemptions for drug donations, voluntary licensing and participation in patent pools would provide positive incentives for bringing patented products to the poor without creating conflict. Countries would forgo some of their tax take if they opted out of the system, thus giving them incentives to honor patents.

Although patent-holders would not welcome such a tax, they might pay it in exchange for a drastic reduction in the scope of pharmaceutical compulsory licenses. Patent-holders would gain security for their intellectual property in exchange for making direct financial contributions to the global public good. Nations, particularly poor ones, could tap into a vast pool of new resources for addressing infectious disease burdens and a growing burden of noncommunicable diseases. As so few countries actually benefit from compulsory licenses as they now stand, they may not be missed.

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**"Compulsory licenses increasingly target costly treatments in middle-income countries."**



## Biotech's roots

**Genentech: The Beginnings of Biotech**

Sally Smith Hughes

University of Chicago Press, 2011

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Reviewed by Phillip A Sharp

Common lore states that there is no history, only biography. Sally Smith Hughes's new book on Genentech is an important addition to the history of biotech. *The Beginnings of Biotech* follows the creation of Genentech by the venture capitalist Robert Swanson and the scientist Herbert Boyer. Although the book tells the story of the birth of biotech from the perspective of Genentech, it is a good read because it focuses on the people and science on the West Coast from which the industry grew.

The story opens with the science of Stanley Cohen and Boyer in the 1970s that led to the highly remunerative patent covering the uses of recombinant DNA. Cohen, an expert in drug-resistant plasmids, and Boyer, an expert in restriction endonucleases, met at a conference in Honolulu in 1972 and decided to collaborate on making recombinant molecules. Their first paper was published in 1973 and led to the subsequent experiment involving John Morrow, a graduate student from Paul Berg's laboratory, that formed the basis for the patent. That Cohen and Boyer got the patent and Paul Berg got the Nobel Prize in Chemistry is an unresolved issue in the book. Hughes focuses on the story behind the patent; it remains for someone else to tell the other story.

Swanson is the hero of Hughes's story. He clearly saw the potential of biotechnology to generate important new products and profits. At the time of the establishment of Genentech, he was recently unemployed but fascinated by recombinant DNA, at least partially through contact with the former company Cetus. A cold call to Boyer and a couple of beers created the first biotech company. Hughes, with passion and sensitivity, tells how Swanson, supported by venture capitalist Tom Perkins, led the struggling new start-up. The first objective was the exclusive right to the Boyer-Cohen patent, which would have blocked the formation of competing companies. This was not successful. The second was demonstrating the power of Genentech's technology. The company started in academic labs at the University of California, San Francisco (UCSF), City of Hope and Caltech. This made for complications later but illustrates the power of collaboration in getting some-

thing done as soon as possible. At this stage, many names linked to the early days of Genentech enter the story, including Robert Crea, Herb Heyneker, Peter Seeburg, John Shine, Axel Ullrich and David Goeddel.

Boyer and Swanson first decided to clone synthetic oligonucleotide sequences for the 14 amino acids of somatostatin and purify the protein from bacterial extracts. The synthetic DNA oligomers were made by Keiichi Itakura of City of Hope and some by Richard Scheller at Caltech. Arthur Riggs, also at City of Hope, worked with the Boyer-led Genentech team to assay the hormone. At the same time, the teams took on the synthesis of human insulin A and B chains. The tension in the story builds as the initial capital decreases and the hormones are not delivered. In the end, Genentech is launched, but some years later City of Hope and Genentech were locked in litigation over the extent of royalties due to the former for the early technology patents from these projects.

Although not mentioned by Hughes because of its minor role and probably the suppressed memories of Swanson, I witnessed a piece of the early moments of Genentech. In 1977, a venture capital firm, INCO, invited me to consult on an external investment in Genentech. At the meeting, Swanson, Boyer, Itakura and Riggs made the pitch in the offices of Kleiner Perkins of producing somatostatin and insulin as the immediate objectives of the company. INCO made the investment, but later Swanson demanded that INCO divest of its Genentech equity when it led the establishment of a competing biotech company, Biogen.

The race to clone the gene encoding insulin is a major part of the story. As it unfolds, Genentech is in negotiations with Eli Lilly and other companies, and Walter Gilbert of Harvard, funded by Biogen, is leading a competing team, and another team at UCSF led by Howard Goodman and Bill Rutter is also in the hunt. Lilly bets on two horses and signs an agreement with both Genentech and UCSF. Another fascinating story is the migrating cDNA clone for human growth hormone. As Hughes tells it, Axel Ullrich and Peter Seeburg took the clone to Genentech after a midnight raid on John Baxter's freezer at UCSF. The question of the importance of this clone in the success of Genentech's cloning of the gene encoding human growth hormone was a matter of litigation that ultimately produced a new building named for Genentech at UCSF.

Hughes's book ends with the amazing initial public offering of Genentech, when the stock opened at \$35 and soared to the mid-\$80s in a minute. This reception set the table for the future funding of biotech by Wall Street. Hughes's book is an important contribution to the history of the field, and I expect that there will be many other contributions. Critics may argue that Hughes does not adequately cover the early origins or the social implications of biotechnology. However, I enjoyed this part of the history of the industry and recommend it to both scientists and laymen.

**COMPETING FINANCIAL INTERESTS**

The author declares no competing financial interests.

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# Cisplatin-induced primordial follicle oocyte killing and loss of fertility are not prevented by imatinib

## To the Editor:

TAp63 $\alpha$  is the predominant member of the Trp53 family (consisting of the tumor suppressors Trp53, Trp63 and Trp73), expressed in primordial follicle oocytes<sup>1</sup> and is essential for oocyte death after genotoxic stress<sup>2</sup>. How DNA damage leads to the activation of TAp63 $\alpha$  is poorly defined. Gonfloni *et al.*<sup>3</sup> reported that inhibition of c-Abl with imatinib (Gleevec), which was designed to inhibit the oncogenic kinase BCR-ABL, protects oocytes from cisplatin-induced killing in mice, and therefore they proposed that c-Abl is crucial for the induction of TAp63-mediated apoptosis. However, the relationships between c-ABL, TAp63 and DNA damage-induced cell death are complex<sup>4,5</sup>, and imatinib itself is actually known to promote apoptosis by inhibiting certain kinases that promote cell survival, such as the stem cell factor receptor, c-Kit<sup>6,7</sup>. Accordingly, imatinib is used clinically for the treatment of not only BCR-ABL<sup>+</sup> chronic myelogenous leukemia but also c-Kit-mutated gastrointestinal stromal tumors<sup>8</sup>. Of particular concern, c-Kit is crucial for oocyte survival, with blocking antibodies to c-Kit causing follicular atresia<sup>9</sup>. We therefore independently explored the impact of imatinib on the response of oocytes to DNA damage induced by cisplatin. We found that imatinib did not protect primordial follicle oocytes from cisplatin-induced apoptosis or prevent loss of fertility in two independent strains of mice.

For *in vivo* analysis, we treated CD1 mice (the same strain of wild-type mice used by Gonfloni *et al.*<sup>3</sup>) at postnatal day 7 (P7) with vehicle (PBS) or imatinib (7.5 mg per kg body weight intraperitoneally (i.p.)) or cisplatin (5 mg per kg body weight i.p.), or both imatinib and cisplatin administered together (as was done by Gonfloni *et al.*<sup>3</sup>). We collected ovaries at P10 and quantified ovarian follicles using established methodology<sup>10,11</sup> (Fig. 1a,b and Supplementary Table 1 and Supplementary Methods). In contrast to the data reported by Gonfloni *et al.*<sup>3</sup>, coadministration of imatinib did not rescue primordial follicles from elimination caused by exposure to cisplatin. As Gonfloni *et al.*<sup>3</sup> did not report the primordial follicle count *per se* (rather, they reported on the composite of the primordial follicle count plus the larger primary follicle count), how they defined primordial follicles in their study is unclear.

We also performed a similar analysis with a second wild-type mouse strain (C57BL/6), with imatinib or vehicle administered 2 h before cisplatin or vehicle. As with the CD1 strain, we observed no rescue of cisplatin-induced oocyte death in the C57BL/6 strain (Supplementary Fig. 1a,b and Supplementary Table 1). In our studies, treatment with imatinib alone increased by nearly fivefold the numbers of pyknotic bodies (oocytes with nuclear fragmentation, a hallmark of apoptosis) observed in ovaries *in vivo* (at P10,  $P < 0.03$ ). This is consistent with *in vivo* proapoptotic activity of imatinib in oocytes (Supplementary Fig. 1c) and the notion that an imatinib-sensitive kinase, most likely c-Kit, is crucial for the survival of female germ cells<sup>12</sup>.

Notably, TUNEL staining confirmed that pretreatment with imatinib did not cause a reduction in the number of apoptotic cells in ovaries of mice exposed to cisplatin for 24 or 48 h (Supplementary Fig. 2). When we analyzed mice treated at P5 or P7 as adults at P49 (Supplementary Table 2) or at 9–11 months of age (Supplementary Table 3), we observed no protection against oocyte killing after treatment with imatinib before injection of cisplatin, compared to treatment with cisplatin alone. We observed similar depletion of primordial follicles in both cisplatin- and imatinib plus cisplatin-treated ovaries at P49 ( $P < 0.05$  both, for cisplatin-treated versus vehicle-treated ovaries and for imatinib plus cisplatin- versus vehicle-treated ovaries; Supplementary Fig. 3). We observed no significant differences in depletion of primary or secondary follicles after treatment with imatinib plus cisplatin versus cisplatin alone ( $P = 0.08$  for both primary and secondary follicles). Representative histologic sections are shown in Supplementary Figure 4a,b (P49 and 9–11 months, respectively). We also performed *in vitro* analysis, with imatinib (10  $\mu$ M) or vehicle added to whole P5 C57BL/6 ovary cultures for 2 h followed by exposure to cisplatin (20  $\mu$ M) or vehicle. After a further 24–48 h in culture, quantification of follicles (which contain oocytes) and TUNEL staining demonstrated no protection afforded by imatinib (Supplementary Figs. 5a,b and 6 and Supplementary Table 4).

To study cisplatin-induced infertility, mice that had been treated at P7 with vehicle, imatinib (7.5 mg per kg body weight i.p.), cisplatin (5 mg per kg body weight i.p.) or both imatinib and cisplatin administered together were studied in mating rounds of approximately 5 weeks, as described in Gonfloni *et al.*<sup>3</sup> from the age of 6 weeks (Supplementary Table 5). We calculated the proportion of pregnant mothers and the average pup number per mating round for the CD1 strain. We observed no difference for the co-administration of imatinib with cisplatin compared with cisplatin alone (Fig. 1c,d). The total number of pups as per Gonfloni *et al.*<sup>3</sup> was no different for mice treated with imatinib and cisplatin versus those treated with cisplatin alone (Fig. 1e and Supplementary Table 5).

In their breeding studies, Gonfloni *et al.*<sup>3</sup> did not provide a statistical analysis in support of their assertion that imatinib preserved fertility of mice exposed to cisplatin. The most stringent measure of reproductive potential is provided by analysis of fertility (the ability to produce a litter within 12 weeks of mating). To study infertility, rather than just the proportion of mice becoming pregnant within the short 5-week mating rounds described by Gonfloni *et al.*<sup>3</sup>, we observed breeders for a total of 12 weeks after previous mating or delivery. Histologic analysis of ovaries from adult females that became sterile during these breeding studies (Supplementary Table 6) confirmed the absence of viable ovarian follicles (Supplementary Fig. 4c). The proportion of females in each treatment group noted to be fertile at each round of

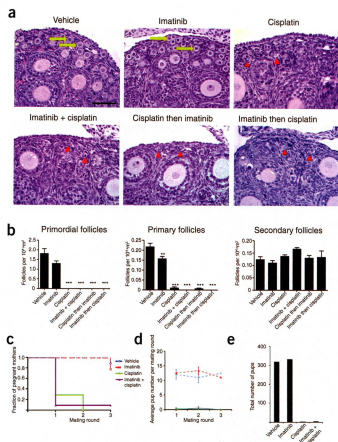


**Figure 1** Pretreatment with imatinib does not protect primordial follicle oocytes from DNA damage-induced death or rescue cisplatin-induced loss of fertility in CD1 mice. P5 CD1 female pups were treated with vehicle (PBS), imatinib (7.5 mg per kg body weight i.p.), cisplatin (5 mg per kg body weight), imatinib and cisplatin administered together, cisplatin administered before imatinib or imatinib administered 2 h before cisplatin. Ovaries were harvested at P10. **(a)** H&E staining of ovaries: vehicle-treated and imatinib-treated ovaries show numerous primordial follicles with oocytes (green arrows). In all cisplatin treatments or different regimens of imatinib plus cisplatin treatments, oocyte-containing primordial follicles are absent, but empty follicle-like structures lacking an oocyte are numerous (red arrowheads). Scale bar, 50  $\mu$ m. **(b)** Quantification of primordial, primary and secondary follicles in CD1 mice treated as above and analyzed at P10. No differences in primary and secondary follicle numbers were observed among groups (not shown). For comparison with untreated controls: \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ,  $n = 3$  ovaries per treatment group. **(c–e)** P5 CD1 female pups were treated with vehicle (PBS), or imatinib (7.5 mg per kg body weight i.p.), cisplatin (5 mg per kg body weight) or imatinib and cisplatin administered together and allowed to mature. Mice commenced breeding trials at P42 with proven wild-type males, and the mating procedure was repeated at regular intervals (about every 5 weeks) as per Gonfioni *et al.*<sup>3</sup>. **(c)** The proportion of cisplatin-treated females becoming pregnant (fraction of pregnant mothers as reported in Gonfioni *et al.*<sup>3</sup>) was not altered by co-administration of imatinib (Kaplan–Meier analysis, cisplatin versus imatinib plus cisplatin  $P > 0.7$ ,  $n = 8–11$  mice per treatment group). **(d)** The average pup number per mating round was not altered by co-administration of imatinib ( $n = 7–11$  pups per breeding round per treatment). **(e)** The total pup number generated as a result of the breedings described above was not altered by coadministration of imatinib ( $n = 319, 334, 4$  and 5 pups, respectively). All animal procedures were approved by the Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee.

breeding and the average number of pups per litter (once the mother became pregnant) were determined. Ovulatory follicles present beyond 6 weeks after treatment were considered to be derived from primordial follicles that survived cisplatin treatment, which indeed is not sterilizing until after three rounds of breeding<sup>3</sup>. In our studies, after allowing female breeders to continue mating for a total of 12 weeks after previous delivery (or until they became pregnant, whichever occurred first, either of these being considerably longer than the 1 week of mating time observed by Gonfioni *et al.*<sup>3</sup>), we observed that treatment with cisplatin at P5–P7 caused a significant deficit in fertility ( $P = 0.02$  PBS versus cisplatin), with only 72% of females remaining fertile by breeding round 6 compared with 100% in the vehicle-treated group. Of note, in up to six rounds of breeding, we found no evidence for rescue of cisplatin-induced loss of fertility by pretreatment with imatinib (Supplementary Fig. 6a and Supplementary Table 6). The likelihood of infertility in each treatment group was no different between females treated with imatinib before cisplatin and those treated with cisplatin alone ( $P > 0.3$  for imatinib before cisplatin versus cisplatin alone, Kaplan–Meier analysis) (Supplementary Fig. 6b). Consistent with this, the average pup numbers seen at each breeding round in litters treated with imatinib before cisplatin were not significantly different from those seen in litters from females treated with cisplatin alone ( $P > 0.6$ ) (Supplementary Fig. 6a).

In conclusion, we have shown that imatinib does not protect primordial follicle oocytes from cisplatin-induced apoptosis and loss of fertility in two independent strains of mice. These results indicate that imatinib-sensitive kinases, such as c-Abl, are not required for the DNA damage-activated oocyte apoptosis that is mediated by Tap63. Indeed, the imatinib-sensitive kinase c-Kit is known to be crucial for the survival of female germ cells<sup>9</sup>, heightening concerns about the

potential effects of imatinib on oocytes and female fertility. Thus, we find no support for “a new use for imatinib, aimed at preserving oocytes of the follicle reserve during chemotherapeutic treatments”<sup>3</sup> and urge caution in this regard.

Note: Supplementary information is available at <http://www.nature.com/dofinder/10.1038/nm.2889>.

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#### AUTHOR CONTRIBUTIONS

J.B.K. and K.J.H. performed and planned experiments, interpreted data and wrote the manuscript. M.C. helped with experiments and contributed data. T.P.S. performed statistical analysis and contributed to manuscript writing. A.S., J.K.F. and C.L.S. conceived of the study, planned experiments, interpreted data and wrote the manuscript.

#### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# Maiani *et al.* reply:

Kerr *et al.*<sup>1</sup> report that imatinib does not protect primordial oocytes from cisplatin-induced apoptosis. This stands in contrast with the results of a recent report from our group in *Nature Medicine*<sup>2</sup>. Furthermore, Kerr *et al.*<sup>1</sup> claim that imatinib causes oocyte death, possibly by blocking the tyrosine kinase activity of the stem cell factor receptor c-Kit, which is considered crucial for oocyte survival<sup>3</sup>. They state that they find no support for a new use for imatinib aimed at preserving the follicle reserve during cancer therapy.

While attempting to reconcile the contradictory outcomes of their study and ours, we noticed that Kerr *et al.*<sup>1</sup> used a hospital-grade cisplatin solution (cisplatin-HG) commonly used in cancer therapy. In comparing the effects on cell line and oocyte survival of cisplatin-HG and Sigma cisplatin (which we used in our original report<sup>2</sup>), we observed that the latter is consistently less effective in inducing cell death if compared to apparently equivalent solutions of cisplatin-HG (Teva, 1 mg ml<sup>-1</sup>) (Supplementary Fig. 1). This may be due to the low solubility of Sigma cisplatin in PBS at concentrations around 1 mg ml<sup>-1</sup>.

Therefore, we repeated some crucial experiments using cisplatin-HG at a dose (2.5 mg per kg body weight) that, according to the titration curve (Supplementary Fig. 1c), induces the same effect caused by 5.0 mg per kg body weight of Sigma cisplatin, the concentration used in our original report. The results of these new experiments confirm that imatinib prevents oocytes of primordial and primary follicles from degeneration induced by cisplatin-HG *in vivo* (Fig. 1a,b). We also clearly observed oocyte protection *ex vivo*, both on ovary fragments cultured in the presence of cisplatin for 24 h (Fig. 1c,d) and on intact gonads, as evaluated by TUNEL staining (Fig. 1e). In addition, as in our original report<sup>2</sup>, we did not observe any significant toxic effect on oocytes by imatinib alone either *in vivo* or *ex vivo* at a similar range of concentrations (or even higher) than those reported by Kerr *et al.*<sup>1</sup> (*in vivo*: 7.5–30 mg per kg body weight Supplementary Fig. 2; *ex vivo*: 1 µM–10 µM, Fig. 1c–e). Notably, imatinib used at therapeutic doses, which are higher than those used in our studies, has no impact on folliculogenesis or spermatogenesis in a leukemia mouse model<sup>4</sup>.

The major conclusion of Kerr *et al.*<sup>1</sup> is that “imatinib-sensitive kinases, such as c-Abl, are not required for the DNA damage-activated oocyte apoptosis that is mediated by Tap63. We disagree with this conclusion. We confirm here that imatinib can protect oocytes challenged by cisplatin in three different experimental settings, as the lack of protection reported by Kerr *et al.*<sup>1</sup> may be only a consequence of excessive oocyte killing caused by higher doses of cisplatin. For additional independent evidence that c-Abl is involved in oocyte degeneration induced by cisplatin, we also tested the ability of GNF-2, a second c-Abl inhibitor<sup>5</sup> (Supplementary Fig. 3), to protect primordial and primary oocytes after cisplatin incubation. GNF-2 works by an allosteric mechanism that is completely independent from imatinib. This molecule targets a deep pocket in the kinase domain, a site far from the ATP-binding site, only when c-Abl is in the auto-inhibited state. GNF-2 has no affinity for tyrosine

kinase receptors such as c-Kit or platelet-derived growth factor<sup>6</sup>. Pretreatment with GNF-2, as we also reported for imatinib<sup>2</sup>, prevented Tap63α accumulation and activation induced by cisplatin in a human Saos-2 p53-null stable cell line expressing Tap63α under the control of a tetracycline-inducible promoter (Supplementary Fig. 4). We observed that GNF-2 can also protect the mouse oocytes from cisplatin-induced death (Fig. 1f,g).

In this new analysis, we adopted a robust methodology to estimate the loss of perinatal oocytes in a large number of untreated and treated gonads. Follicles were identified exclusively by immunohistochemistry for an antigen specific for germ cells (Mys2) and by the number of layers surrounding the oocyte. This analysis relies on a clear-cut staining procedure that, while facilitating the counting of viable oocytes, at the same time allows inclusion in the counting a higher number of mid-ovary slices for each gonad (approximately ten sections per ovary; we counted one section in every three). We adopted a previously reported procedure<sup>7</sup> that focuses on the population of follicle (primary and primordial) expressing high amounts of Tap63α. Follicle classification and counting considering only two populations is less error prone and is sufficiently informative for the purpose. In addition, within the population of reserve oocytes rescued by the treatment with imatinib, we did not find a predominance of primary follicles versus primordial ones, as also clearly illustrated in Figure 1a,f and in Supplementary Figure 5.

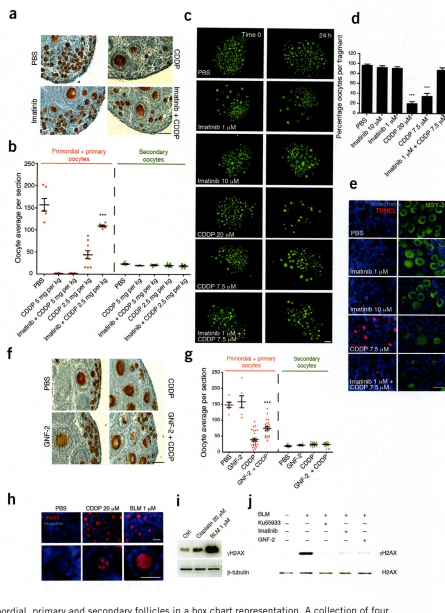
Given that Kerr *et al.*<sup>1</sup> used doses of cisplatin resulting in oocyte degeneration that cannot be rescued by imatinib, it is not surprising that, in the mating experiments, they could not show any preservation of fertility. On the one hand, we accept that a larger number of mice than used in our initial study should be considered before reaching solid conclusions. On the other hand, the number of mice we used was clearly reported in our paper<sup>2</sup>. At the moment, consistent with our report that fertility is prolonged by imatinib in cisplatin-treated mice<sup>2</sup>, we can confirm that, after treatment with appropriate doses of cisplatin, oocytes of the follicle reserve at P42 are much more abundant in ovaries pretreated with GNF-2 (Supplementary Fig. 6).

Finally, we show that pharmacological inhibition of c-Abl can have an impact on the DNA damage signaling response at a very early stage. We found that preincubation with either imatinib or GNF-2 prevents H2AX phosphorylation on Ser139 (indicated as γ-H2AX), an early marker of the DNA damage response (DDR). We analyzed whole-ovary cultures exposed to either CDDP or bleomycin, a radiomimetic compound, by means of immunofluorescence and western blotting. Notably, bleomycin could induce a massive phosphorylation of H2AX on Ser139 (Fig. 1h–j) within a short time interval. Treatment with either c-Abl inhibitor resulted in a drastic reduction of Ser139 phosphorylation in H2AX. As a control, we also tested a potent inhibitor (Ku55933) of ATM, a PI3K-related kinase mainly involved in Ser139 phosphorylation of H2AX following DNA damage. The effect of Ku55933 mirrored those obtained with both c-Abl inhibitors (Fig. 1j).

Recent evidence supports a role of c-Abl in the early stage of the DDR<sup>8,9</sup>. Several studies have placed H2AX post-translational modifications at the center stage of DDR paths. These modifications are



**Figure 1** Pharmacological inhibition of c-Abl protects reserve follicle oocytes and impairs  $\gamma$ H2AX phosphorylation induced by DNA damage. Postnatal day 7 (P7) CD1 female pups were treated with vehicle (PBS), imatinib (Novartis, 7.5 mg per kg body weight) or GNF-2 (Sigma, in a range of 8.0–9.5 mg per kg body weight). After pretreatment (4 h), mice received a single dose of cisplatin-HG (Teva, equal volume of 1 mg  $\text{ml}^{-1}$  solution) (range of quantity 2.5–3.0 mg per kg body weight). At P10, mice were killed and ovaries were analyzed by immunohistochemistry. (a) Ovaries from P10 mice treated with PBS, imatinib or cisplatin-HG (CDDP) alone or in combination with imatinib. Ovary sections were stained with MSY-2-specific antibodies (brown) and hematoxylin (blue). Scale bar, 25  $\mu\text{m}$ . (b) Quantification of primordial, primary and secondary follicles in a box chart representation. A data point represents the average number of primordial and primary or secondary follicles derived from approximately ten sections counted per single gonad. Means  $\pm$  s.e.m. are reported. For comparison with cisplatin-treated mice, the analysis of variance was carried out as described in the **Supplementary Methods**: \*\*\* $P < 0.0001$ . (c) Ovary fragments from p70 c-Ki/GFP mice<sup>13</sup> were treated, after 3 d of culture, for 24 h with cisplatin and imatinib. Imatinib was added 3 h before CDDP. GFP+ oocytes with a diameter  $\geq 18$ –20  $\mu\text{m}$  were counted at time 0 and after 24 h of culture. Scale bar, 25  $\mu\text{m}$ . (d) Percentage of surviving oocytes scored in each fragment after 24 h of culture. At least three fragments were scored for each experimental condition. Data represent the mean values  $\pm$  s.e.m. of three independent experiments. (e) Sections of ovaries from P7 mice cultured for 36 h with or without CDDP or imatinib stained with TUNEL (red) and for MSY-2 (green). When CDDP and imatinib were added together, a preincubation of 3 h with imatinib was performed before adding cisplatin. Scale bar, 25  $\mu\text{m}$ . (f) P7 CD1 mice were injected with vehicle (PBS) or GNF-2 and 4 h later treated with cisplatin-HG. (g) Ovary sections from P10 mice treated as indicated were stained with MSY-2-specific antibodies (brown) and hematoxylin (blue). Scale bar, 25  $\mu\text{m}$ . (h) Quantification of primordial, primary and secondary follicles in a box chart representation. A collection of four independent experiments is plotted in the figures. Means  $\pm$  s.e.m. are reported. For comparison with cisplatin-treated mice, an analysis of variance was carried out as described in the **Supplementary Methods**: \*\*\* $P < 0.001$ . (i) Top, ovaries cultured for 36 h with CDDP or for 2 h with bleomycin were stained with H2AX-specific antibody (red). Scale bar, 25  $\mu\text{m}$ . Bottom, oocyte magnification showing  $\gamma$ H2AX foci. Scale bar, 25  $\mu\text{m}$ . (j) Western blots of phosphoH2AX (Ser139) and tubulin in ovary extracts after cisplatin (Sigma, dissolved in DMSO) or bleomycin (BLM) treatment. (k) Western blots of phospho-H2AX and H2AX in ovary extracts after a short exposure (20 min) to bleomycin (2  $\mu\text{M}$ ) alone or in combination with different kinase inhibitors as indicated (10 min of preincubation with GNF-2 at 14  $\mu\text{M}$ , imatinib at 10  $\mu\text{M}$  or Ku55933 at 10  $\mu\text{M}$ ). Mouse studies were approved by the Institutional Animal Care and Use Committee of Tor Vergata University.



important for recruiting DNA damage proteins and for the amplification, through ubiquitin-dependent signaling, of the DNA damage cascade<sup>10–12</sup>.

In conclusion, our new data confirm the role of c-Abl in the induction of oocyte degeneration after DNA damage. We conclusively demonstrate the involvement of c-Abl at an early time point of the DDR, which is reflected at later times in oocyte degeneration. By using two inhibitors that work by orthogonal mechanisms, we have also ruled out alternative explanations that invoke the cross reactivity of imatinib with different kinases.

Note: Supplementary information is available in the online version of the paper.

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#### AUTHOR CONTRIBUTIONS

E.M. did mouse injections, immunohistochemistry, statistical analysis of data and *ex vivo* bleomycin experiments; C.D.B. helped with *in vivo* and *ex vivo* experiments;

F.G.K. made ovary fragments and did TUNEL; S.M.C. and S.B. did follicle counting; S.C. did FACS analysis; F.M. did cell culturing; M.M. provided support for *in vivo* experiments; M.D.F. contributed to scientific discussions; M.D. provides support to complete this study; G.C. provided support and critical reading of the manuscript; S.G. designed the study, set up the immunohistochemistry-based approach, supervised the project and wrote the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# TRP-ing up brain tumors

David L Schonberg, Shideng Bao & Jeremy N Rich

**Malignant gliomas are devastating, uniformly fatal cancers for which current therapies remain palliative. A new study in mice shows that neural precursor cells, abundant in neonatal brains, release a fatty acid factor that induces glioma cell death through the activation of TRPV1 channels, prolonging survival and potentially uncovering a new treatment strategy (pages 1232–1238).**

Malignant gliomas—including anaplastic gliomas (World Health Organization (WHO) grade III) and glioblastomas (WHO grade IV)—are the most prevalent primary brain tumors in adults<sup>1</sup>. Despite aggressive multimodal therapy, including maximal surgical resection, combined radiation and chemotherapy, and adjuvant chemotherapy, the prognosis of glioblastoma remains dismal, as most people diagnosed with the disease die within 12–15 months<sup>1</sup>. Cytokine-based vaccines, active cell-based immunotherapies (many in phase 1 or 2 clinical trials) and targeting of the neovasculature with the US Food and Drug Administration–approved anti-vascular endothelial growth factor angiogenesis inhibitor, bevacizumab, have yielded modest results<sup>2</sup>. The minimal incremental benefit of chemotherapy and targeted therapies has prompted the development of cell-based (immunologic and stem cell) therapies (Fig. 1). Stem cells and neural precursor cells (NPCs) have been used as delivery vehicles for gene therapy in animal models, as they have tropism for tumor cells when implanted into experimental intracranial gliomas, but they have not been therapeutic by themselves<sup>3</sup>. However, transplanting stem cells or NPCs into the brains of patients poses considerable hurdles, and harnessing the innate tumor-suppressive ability of these cells into efficacious therapy remains relatively unexplored.

In this issue of *Nature Medicine*, Stock *et al.*<sup>4</sup> show that NPCs in the juvenile brain

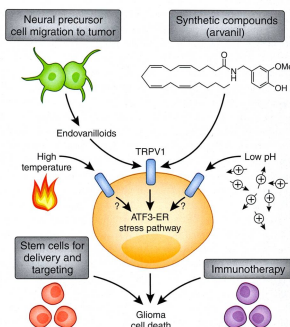


**Figure 1** NPCs induce glioma cell death via TRPV1 channels. NPC-derived endovanilloids stimulate TRPV1 channels, leading to increased ATF3 expression and activation of the ER stress pathway, resulting in glioma cell death. The synthetic vanilloid arvanil elicits similar tumor-suppressive effects. Changes in pH and temperature can also activate TRPV1 channels, yet it is unknown whether these signals activate the same ER stress response in glioma cells. Current therapeutic approaches for brain tumors include targeting cancer stem cells and using neural, mesenchymal, hematopoietic, cord blood and embryonic stem cells as delivery vehicles. Enhancing tumor-suppressive capabilities from endogenous cells, such as neural precursor cells, has clinical advantages and may be applicable to a variety of tumors. Immunotherapy includes tumor-antigen sensitization (active immunotherapy), cytokine modulation (passive immunotherapy), and ex vivo activation, followed by re-infusion of patients' immune cells (adoptive immunotherapy).

use a new mechanism to fight malignant gliomas, which could be exploited to develop molecules to target this same pathway in adult brain tumors. The authors show that NPCs migrate to gliomas and suppress tumor growth by releasing fatty acid ethanolamides, called endovanilloids, with antitumor activity<sup>4</sup>. These factors showed agonistic ability on the vanilloid nociceptor transient

receptor potential cation channel subfamily V member 1 (TRPV1), which is highly expressed in malignant gliomas. TRPV1 stimulation on tumor cells *in vitro* induced cell death through the activating transcription factor 3 (ATF3)-mediated endoplasmic reticulum (ER) stress pathway (Fig. 1). Notably, Stock *et al.*<sup>4</sup> were able to mimic NPC-mediated tumor suppression in adult, tumor-bearing mouse

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brains by systemic treatment with the synthetic vanilloid arvanil. This study<sup>4</sup> and an earlier study<sup>5</sup> show that TRPV1 agonists such as arvanil show preclinical efficacy against malignant gliomas, particularly glioblastomas. It is interesting that the authors find that even conditioned medium from endogenous NPCs may be cytotoxic to glioma cells, in contrast to other reports in which exogenous NPCs and other stem cells track but do not kill glioma cells<sup>6</sup>.

Whereas TRPV1 expression has been reported to inversely correlate with glioma grade<sup>7</sup>, Stock *et al.*<sup>4</sup> detected the highest TRPV1 expression in glioblastoma cells. TRPV1 is predominantly localized in the neoplastic compartment compared to the normal neural parenchyma. Therefore, the therapeutic index for vanilloids is potentially high, given their specificity to tumors and limited side effects when delivered to the brain. Exogenous NPCs are highly attracted to tumor cells<sup>3,6</sup>, a finding translated to an active clinical trial involving patients with malignant glioma<sup>8</sup>, and TRPV1 modulation may therefore augment the efficacy of NPC-based therapy for adults in whom NPCs are depleted or not functional due to aging. In addition to changes in frequency, the microenvironment and cell-autonomous characteristics of NPCs also change with age<sup>9</sup>. This may explain why the NPC-induced tumor suppression described by Stock *et al.*<sup>4</sup> is age dependent, as only young mice orthotopically injected with glioma cells are sensitive to TRPV1 knockdown. These findings are surprising in light of the frequent use of very young mice as hosts in genetically engineered glioma models and xenograft studies, suggesting that juvenile NPCs are not universally antitumorigenic.

One could postulate that the greater number of NPCs found soon after birth would thwart the development of pediatric brain tumors. Sadly, this is not the case, as pediatric brain tumors (including gliomas, medulloblastomas and ependymomas) are the leading cause of childhood cancer deaths (<http://www.cbtrus.org/factsheet/factsheet.html>). Pediatric brain tumors often coincide with postnatal neurogenesis and NPC activity. Further, these cancers are fundamentally different from those in the adult brain, in terms of tumor type (for instance, medulloblastomas are rare in adults), location, genetic abnormalities and response to therapy<sup>10,11</sup>. Future studies will be required to determine whether intrinsic

properties that distinguish pediatric from adult high-grade glioma cells differentially regulate NPC-induced tumor suppression. In addition, it is unclear whether TRPV1 expression and function in pediatric brain tumors are consistent with that described by Stock *et al.*<sup>4</sup> in adult tumors, which may affect the development of TRPV1 agonists for treatment of brain tumors across all cases.

It is intriguing to speculate as to why NPCs secrete much higher concentrations of endovanilloids than differentiated or neoplastic cells. Furthermore, what is the function of nociceptive receptors such as TRPV1 on tumor cells, specifically on highly invasive and resistant cells (typical of high-grade gliomas)? In addition to endovanilloids, TRPV1 channels are also sensitive to changes in pH and temperature (Fig. 1), two microenvironmental components that can regulate tumor growth<sup>12,13</sup>. Solid tumors have considerably lower pH than normal tissue, and tumor acidity has been shown to increase angiogenesis and promote self-renewal and tumorigenicity of glioma stem cells<sup>14</sup>. Likewise, TRPV1 channels also respond to increases in temperature, and numerous studies have investigated the therapeutic benefit of applying direct hyperthermia to high-grade gliomas. These alternative TRPV1-activating signals may provoke the same ER stress response in glioma cells that is observed with endovanilloids. Additionally, TRPV1-mediated glioma cell death may be more prominent in specific tumor microenvironments such as hypoxic and perivascular niches and/or cellular subtypes (that is, proneural versus mesenchymal) that have been shown to be resistant to current therapy.

The cytotoxic effects of NPC-secreted endovanilloids bring into question whether glioma cells repeatedly exposed to endovanilloids will eventually become desensitized to subsequent stimuli, which can occur with TRPV1 receptors and would potentially result in diminished TRPV1-mediated cell death. Perhaps stimulating TRPV1 in glioma cells with exogenous vanilloids, such as the synthetic arvanil, activates ER stress before desensitization can occur. Stock *et al.*<sup>4</sup> show that arvanil increases survival of mice receiving orthotopic human primary glioblastomas, including those mice non-responsive to temozolomide treatment, an oral alkylating chemotherapy now the standard of care for high-grade gliomas. TRPV1

agonists may be a new addition to brain tumor therapy, especially because it seems that normal, nonpathological tissue is unaffected by exogenous vanilloids<sup>4</sup>. Caution must be taken, however, as it is possible that the function of TRPV1 in gliomas might be stimulus specific and, depending on the mode of activation, may have a larger role in promoting tumor growth. Nevertheless, the TRPV1 family may become important molecular targets in neuro-oncology, as TRPV2 expression and activity impair glioma stem cell proliferation and promotes differentiation<sup>15</sup>.

The current study may add a new paradigm to the development of brain tumor therapies through the integration of lessons from endogenous antitumor cellular activity into pharmacologic intervention (Fig. 1). There may be additional opportunities and complexities in the translation of these studies to clinical trial, as gliomas are now recognized as a diverse set of tumors with striking differences in tumor growth and response to therapy based on patient characteristics (for example, age), tumor cell of origin, molecular features and prior genotoxic therapy requiring patient selection to optimize efficacy. Although it is unlikely that a single mechanism will empower the endogenous system to completely eradicate the lethality of brain tumors, stem cell induction of tumor cell death through the stem cell's secretory production provides new opportunities for translational research and drug development for currently untreatable diseases.

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The authors declare no competing financial interests.

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# Nine lives for T<sub>H</sub>9s?

Weiping Zou & Nicholas P Restifo

There is currently much interest in dissecting the mechanisms of tumor immunity. A new study shows that a subset of CD4<sup>+</sup> T cells that produce the cytokine interleukin-9 (IL-9) mediate inhibition of melanoma growth in mice and that analogous IL-9-producing T cells are present in human skin (pages 1248–1253). Could such cells be manipulated to develop new therapeutic strategies for melanoma?

Given the myriad and mutable nature of the challenges they face, it seems easy to imagine why T cells would evolve to acquire a diversity of epigenetic states. T helper (T<sub>H</sub>) cells need to be both specific and plastic, but ascertaining the qualities of these states and their precise roles in immune homeostasis is an enormous challenge for modern-day immunobiologists. Production of IL-9 by T<sub>H</sub>2-like cells has been known for some time, but the possibility that this cytokine is produced by a distinct T<sub>H</sub> cell subset—dubbed T<sub>H</sub>9 cells—has gained substantial currency in recent years<sup>1</sup>. T<sub>H</sub>9 cells have been linked to T<sub>H</sub>2 responses and mast cell biology<sup>1,2</sup>, and, in this issue of *Nature Medicine*, Purwar *et al.*<sup>3</sup> ascribe a new role for IL-9 and T<sub>H</sub>9 cells in the protective antitumor immune response.

T<sub>H</sub>9 cells join an ever-growing list of what seem to be metastable epigenetic states of T<sub>H</sub> cell development (Fig. 1). The role of T<sub>H</sub>9 cells in tumor immunity had not been previously investigated in mice or humans, but Purwar *et al.*<sup>3</sup> inadvertently tackled this problem. While attempting to examine the role of retinoid-related orphan receptor- $\gamma$  (ROR- $\gamma$ ), a key transcription factor in the development of the T<sub>H</sub>17 cell lineage, in antitumor immunity, the authors found increased IL-9 expression in ROR- $\gamma$ -deficient T cells. Moreover, mice lacking ROR- $\gamma$  (ROR- $\gamma^{-/-}$  mice) showed reduced tumor growth after subcutaneous injection of melanoma cells compared with ROR- $\gamma^{+/+}$  mice. Hypothesizing that T<sub>H</sub>9 and IL-9 played a part in the protective antitumor immunity they observed in the ROR- $\gamma^{-/-}$  mice, the authors employed several complementary approaches to test this possibility<sup>3</sup>. They found that administration of a monoclonal neutralizing antibody (mAb) against IL-9 promoted melanoma growth in ROR- $\gamma$ -deficient mice, IL-23 receptor-deficient mice and wild-type mice.

To examine the role of different T<sub>H</sub> effector subsets in melanoma immunity (Fig. 1), Purwar *et al.*<sup>3</sup> adoptively transferred *in vitro*-polarized T<sub>H</sub>1, T<sub>H</sub>2, T<sub>H</sub>9 and T<sub>H</sub>17 cells specific for ovalbumin peptide-expressing B16 melanoma into syngeneic immunocompetent mice. Consistently with previously published studies, T<sub>H</sub>17 cells mediated potent antitumor immunity<sup>4–9</sup>. The effects of T<sub>H</sub>9 cells were superior to the other effector T cells in this tumor protection model<sup>3</sup> although it remains unclear from this study what role, if any, T<sub>H</sub>9 cells have in the treatment of established vascularized tumors. In support of a protective role of IL-9, T<sub>H</sub>9 cells or both in tumor immunity, the authors showed that melanoma growth was accelerated in IL-9 receptor-deficient mice compared to heterozygotes for the IL-9 receptor. Furthermore, recombinant IL-9 treatment

impaired tumor growth in vaccine and nonvaccine settings. Therefore, IL-9 and/or T<sub>H</sub>9 cells inhibit tumor growth, at least in the setting of tumor implantation.

To gain mechanistic insights into how IL-9-producing T cells might inhibit the implantation of experimentally transplanted melanoma, the authors studied T<sub>H</sub>9 cells *in vitro*, finding that T<sub>H</sub>9 cells were capable of direct tumor lysis, which was granzyme B dependent<sup>3</sup>. Furthermore, IL-9 administration reduced tumor growth in Rag1-deficient mice, which lack B and T cells, but had no effects on tumor growth in mast cell-deficient mice. These data suggest that tumor growth inhibition mediated by IL-9 depends on the presence of mast cells but not T or B cells. However, the relative impact of mast cells and direct cytotoxicity, or a possible involvement of natural killer cells, in mediating

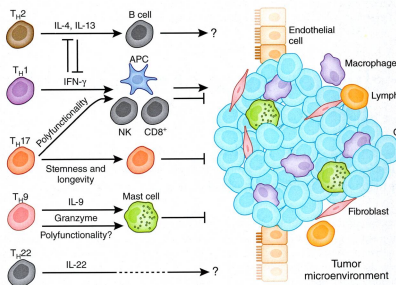


**Figure 1** T helper subsets and tumor immunity. Different T helper subsets are found in tumor microenvironments. The roles of these T cell subsets in tumor immunity may be highly context dependent. T<sub>H</sub>1 cells may mediate antitumor immunity through the interferon- $\gamma$  (IFN- $\gamma$ ) signaling pathway. T<sub>H</sub>2 cells may suppress antitumor immunity by inhibiting the T<sub>H</sub>1 response and inducing alternative macrophages through IL-4 and IL-13. However, it is also possible that T<sub>H</sub>2 cells may promote tumor immunity through B cell activation. The work of Purwar *et al.*<sup>3</sup> suggests that T<sub>H</sub>9 cells may mediate antitumor immunity via the direct killing of tumor cells and by mast cells. The polyfunctionality, stemness and longevity of T<sub>H</sub>17 cells may be associated with their antitumor immunity. The role of T<sub>H</sub>22 cells in tumor immunity is poorly understood, although they may be associated with poor outcome in patients with gastric cancer and have been linked to hepatocellular carcinoma progression. APC, antigen-presenting cell; NK, natural killer cell.

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the antitumor effects of IL-9 still remain to be addressed in future studies (Fig. 1).

In correlative studies, the authors identified  $T_H9$  cells in healthy skin in humans as well as in tumor tissues from individuals with melanoma<sup>3</sup>. They also found that T cells from metastatic melanoma produced less IL-9 than that extracted from healthy donors. These early data may stimulate additional work designed to assess a role for  $T_H9$  cells in tumor progression.

Whether there is a causal relationship between ROR- $\gamma$  deficiency and IL-9 production and  $T_H9$  development remains unexplored. However, given that the transcription factor PU.1 is required for the development of  $T_H9$  cells<sup>10</sup>, it may be interesting to investigate whether ROR- $\gamma$  deficiency biochemically and functionally affects transcription events downstream of PU.1.

Many questions remain to be answered. It is known that the development of both  $T_H9$  and  $T_H17$  cells may be heavily influenced by the transforming growth factor- $\beta$  (TGF- $\beta$ ) signaling pathway, but to what extent are  $T_H9$  molecular and functional programming similar to or different from the unique features of  $T_H17$  cells?  $T_H17$  cells can induce potent tumor immunity, and this may be due to the polyfunctionality and plasticity of these cells<sup>7,8</sup>. Perhaps more importantly,  $T_H17$  cells have the elusive qualities of 'stemness' and longevity<sup>8,9</sup>. The hypoxia-inducible

factor-1 $\alpha$ , Notch and Bcl-2 signaling pathways are important for maintaining the  $T_H17$  cell pool<sup>8,11,12</sup>, but to what extent do  $T_H9$  cells possess these evolutionarily conserved 'stem cell-like' pathways?

In human epithelial cancers,  $T_H17$  cells constitute a small population compared with other effector T cells<sup>5,11</sup>. Intratumoral  $T_H17$  number and/or IL-17 abundance correlate with improved survival in multiple human cancers<sup>4</sup>. High amounts of TGF- $\beta$ , IL-6 and IL-1, which should promote  $T_H17$  cell development, have been detected in the tumor microenvironment<sup>13</sup>. However,  $T_H17$  cells are tightly regulated and suppressed by regulatory T cells in the tumor microenvironment<sup>4,7,14</sup>. Given that Purwar *et al.*<sup>3</sup> observed that the numbers of  $T_H9$  cells were lower in advanced melanoma, similar to what has been observed for  $T_H17$  cells, are  $T_H9$  cells also inhibited by regulatory T cells? In addition to melanoma, are  $T_H9$  cells capable of invading the microenvironments of other human cancers?

Adoptive T cell therapy can induce tumor regression and result in objective clinical responses in patients with cancer<sup>15,16</sup>. The work of Purwar *et al.*<sup>3</sup> may imply that IL-9 and  $T_H9$  cells have a role in immune-based anticancer therapy, but the uses of  $T_H9$  cells in tumor treatment remain unresolved in the present study, where treatment of tumors is performed at the time of their implantation. Future work

will be required to determine whether  $T_H9$  cells have the 'staying power' to eradicate established tumors. As we approach a new set of potent new T cell-based immunotherapies, translational immunologists will need to ascertain if  $T_H9$  cells have the qualities to eradicate swiftly growing tumor cells in an immunosuppressive and hypoxic tumor microenvironment, qualities that are not assessed by 'day 0' treatment models. Could  $T_H9$  cells have the feline qualities of 'nine lives'?

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## The elusive source of myofibroblasts: problem solved?

Jeremy S Duffield

**Fibrosis is omnipresent and contributes to a substantial proportion of all natural deaths. A recent study (pages 1262–1270) provides evidence that the mysterious perivascular cell, also known as the pericyte, is the cell type responsible for fibrotic disease in skin and skeletal muscle.**

Fibrosis has emerged from something pathologists comment on in passing to a central but incompletely understood process of disease. Fibrosis is widespread in many common diseases with limited treatment options, including cancer, cardiovascular diseases, chronic lung and kidney diseases, liver cirrhosis, inflammatory bowel diseases, muscular atrophy and skin diseases such as scleroderma<sup>1</sup>.

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Moreover, fibrosis is now recognized as a process of aging in all organs. There are currently few therapies to treat fibrosis, but at the flagship scientific meeting on fibrosis recently hosted by Keystone Symposia, representatives from 65 pharmaceutical and biotechnology companies committed with renewed gusto to identify therapies to treat this disease process and to bring those therapies to market.

One major problem that has confounded the field(s) has been the lack of understanding of the biological processes involved in fibrosis. However, recent studies, including one by Dulauroy *et al.*<sup>2</sup> in this issue of *Nature Medicine*, may draw the attention of

researchers to the discrete and poorly appreciated populations of mesenchymal cells that are wrapped around capillaries<sup>3</sup>. These cells have been variously called perivascular cells, mural cells, pericytes or pericyte-like cells, but relatively little is known about their normal functions. Moreover, substantial controversy persists around their name, as the cells have been defined by their morphology or appearance under electron microscopy rather than by cell function, ontogeny or transcription factor activation<sup>3,4</sup>.

Dulauroy *et al.*<sup>2</sup> used elegant genetic studies in mice to map the fate of neural crest cell-derived embryonic mesenchyme,

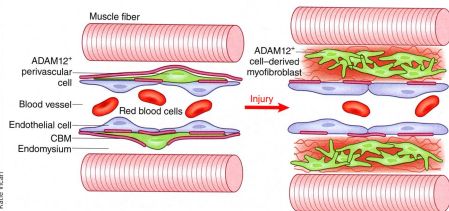


**Figure 1** Mesenchymal perivascular cells are skin and muscle myofibroblast progenitors. Dulauroy *et al.*<sup>2</sup> show that in steady-state conditions, perivascular cells of the ADAM12<sup>+</sup> lineage are embedded in the capillary basement membrane (CBM, dark pink) and attached to endothelial cells. In response to injury or wounding, these pericytes detach from capillaries, migrate into the interstitial space where they become activated, proliferate and deposit pathological fibrillar pathological matrix in a process known as fibrosis. In doing so, capillaries lose the supportive function of the pericytes, as when pericytes become myofibroblasts they lose normal pericyte functions. Such a model of fibrogenesis may also be recapitulated in many internal organs.

which expresses an enzyme, A disintegrin and metalloprotease 12 (ADAM12). They found that the ADAM12<sup>+</sup> cells migrate in early embryogenesis to gut, brain, skin and somites and give rise to several cell populations, including nerves and glial cells<sup>2</sup>. However, a subpopulation of the cells derived from the ADAM12<sup>+</sup> lineage express platelet-derived growth factor receptor- $\beta$  (PDGFR $\beta$ ) and wrap around capillaries in both skeletal muscle and skin, localizing within the capillary basement membrane. By using this fate-mapping approach in skin- and muscle-wounding experiments, the authors showed that the perivascular cells derived from the ADAM12<sup>+</sup> lineage—which express markers of pericytes previously reported in pericytes from brain and eye capillaries—detach, migrate and activate to become myofibroblasts. Myofibroblasts, in a dynamic process, lay down pathological fibrillar fibrogenic matrix, contracting and distorting the cellular architecture of the injured tissue.

Dulauroy *et al.*<sup>2</sup> carried out several carefully designed experiments that support and strengthen their central observation. They found that the specific ablation of ADAM12<sup>+</sup> cells decreased fibrosis, confirming the central role of the perivascular cells derived from the ADAM12<sup>+</sup> lineage in fibrogenesis after wounding<sup>2</sup>. The authors also carried out a parabiotic experiment in which the circulation of one mouse was joined to that of a second mouse<sup>2</sup>. The first mouse had distinct cellular markers, and the second mouse received skin or muscle wounds; the authors assessed the contribution of cells from the first mouse (via the circulation) to the wound<sup>2</sup>. They found that all of the

myofibroblasts that derived from ADAM12<sup>+</sup> cells were from the second mouse<sup>2</sup>. Therefore, the myofibroblasts do not derive from a circulating cell, but are permanently resident in the injured tissue.

Placing these findings in a historical context, in 1970 Russell Ross performed a similar parabiotic experiment to show that myofibroblasts (platelet-derived cells that at that time by electron microscopy) in skin wounds did not derive from a circulating cell, and in 1963 Hermes Grillo proposed that myofibroblasts in skin wounding derived from poorly defined perivascular cells<sup>5,6</sup>. The new study by Dulauroy *et al.*<sup>2</sup> builds on those early studies by defining myofibroblast progenitors at molecular, functional and structural levels. The findings of Dulauroy *et al.*<sup>2</sup> may also put to rest some of the controversies of the origins of myofibroblasts that have been debated over the last 20 years; in particular, the question of whether a circulating fibrocyte, possibly of myeloid origin, is a major contributor to fibrogenesis in skin wounding.

How do the new findings of Dulauroy *et al.*<sup>2</sup> marry with the fibrogenic process in our central organs? Studies of liver fibrosis dating back to 1985 identified a specialized mesenchymal cell, which was given several names, including the Ito cell and the lipocyte, and which was thought to be the precursor of myofibroblasts in liver disease. This cell, now known as the hepatic stellate cell, is the pericyte-like cell of the hepatic sinusoid, and is important for maintaining the fenestrated endothelium of this specialized capillary. Hepatic stellate cells have long been suggested as the primary myofibroblast progenitors in

the liver, and this theory has recently been confirmed by genetic fate-mapping studies in mouse models of alcoholic and toxic liver fibrosis<sup>7,8</sup>. Over the last two years similar mesenchyme-derived perivascular cells have been shown to be major myofibroblast progenitors in kidney diseases, central nervous system injury, intestinal injury and lung fibrosis<sup>9–11</sup>. It may therefore be the case that discrete mesenchymal cells, which are wrapped around capillaries, are present throughout our tissues. These cells may respond to injury or wear-and-tear by activating, detaching from the capillary and becoming pathological matrix-depositing cells. Such a scenario could present us with a unifying model of fibrogenesis (Fig. 1).

Many questions arise from the work of Dulauroy *et al.*<sup>2</sup>, as it applies specifically to the development, homeostasis or disease of muscle and skin. What is the function of pericytes? As in the central nervous system, eye and kidney, do they regulate angiogenesis, vascular stability, permeability and flow<sup>12</sup>? And are these functions lost when pericytes become myofibroblasts, leaving unstable capillaries? Are they cancer suppressor cells? As pericytes can regenerate capillaries and may serve as progenitor cell niches for muscle satellite cells, is fibrogenesis due to pericyte-to-myofibroblast transition in skeletal muscle a state of satellite cell incompetence? Perivascular cells may harbor their own intrinsic progenitor cell function as they have the capacity to generate adipocytes in skin. Is there a dynamic relationship between changes in adipose tissue and fibrogenesis via the pericyte, and do the observations made in skin wounding translate to the chronic skin disease scleroderma? Because the lineage of pericytes in skin differs from that of liver and kidney, is there an early single progenitor of all mesenchymal cells in the body, or are there truly distinct lineages with distinct properties?

From a drug-development perspective, such a unifying model of fibrogenesis focused on the perivascular cell would provide a central cellular target with a stereotyped molecular repertoire and responses to signals. What may turn out to be more challenging will be how to limit deleterious myofibroblast functions while preserving healthy perivascular cell functions.

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## Febrile seizures and the wandering granule cell

Rod C Scott & Gregory L Holmes

**Prolonged febrile seizures in young children have long been suspected to lead to temporal lobe epilepsy, but how this occurs has been unclear. A new study (pages 1271–1278) in rats showing that febrile seizures induce aberrant migration of cells in the temporal lobe suggests this may be a crucial component in the development of epilepsy.**

Febrile seizures are the most common of all childhood seizures, occurring in 2–5% of children between ages 6 months and 6 years. About a third of children with febrile seizures will have recurrences. The seizures are always provoked by fever, are short (<5 min) and are inconsequential with regard to causing brain damage or subsequent epilepsy. However, approximately 10% of children that develop febrile seizures will have febrile status epilepticus, a seizure caused by fever lasting greater than 30 min<sup>1</sup>. Whether long febrile seizures in children with otherwise normal brains are also benign is far less clear. The observation that many individuals with temporal lobe epilepsy (TLE) have a history of long febrile seizures without additional risk factors for epilepsy such as head trauma, brain malformations or stroke has raised the concern that febrile seizures adversely and permanently alter the developing brain<sup>2</sup>.

A rodent model of experimental febrile seizures (EFSs) has been used to study the relationship between prolonged febrile seizures and the subsequent development of epilepsy. In this model, hyperthermia in rat pups induced by raising the ambient temperature and increasing core body temperatures over 40 °C results in generalized seizures<sup>3</sup>. After EFSs lasting ~24 min, a considerable portion of rats will develop spontaneous temporal lobe seizures and cognitive impairment<sup>3,4</sup>. Understanding how EFSs lead to epilepsy is of considerable clinical importance, given the debate as to whether a prolonged febrile seizure leads to TLE or whether the

febrile seizure occurs as a result of a congenital temporal lobe abnormality (such as dysplasia that predisposes the child to a prolonged febrile seizure and subsequent TLE). Thus far, there is no known therapy that has been shown to prevent TLE after prolonged febrile seizures.

The dentate gyrus, part of the hippocampal formation in the temporal lobe, has neurogenesis throughout life and is a major gateway into the hippocampus receiving input from neocortex (Fig. 1). Neonatal-generated granule cells, neurons that reside in the dentate gyrus and normally migrate from the subgranular zone to the granular cell layer of this hippocampal formation, use axons called mossy fibers to project to principal pyramidal neurons in the hippocampal region called CA3, forming an excitatory pathway involved in learning and memory formation. Most individuals with TLE after prolonged febrile seizures have abnormalities in the hippocampus (Fig. 1). In this issue of *Nature Medicine*, Koyama et al.<sup>5</sup> show that EFSs in rat pups cause an aberrant migration of granule cells. Remarkably, EFSs cause these granule cells to end up in the hilus rather than the dentate gyrus, causing enhanced excitation of the migrating cells. The rats, when studied as adults, had epilepsy, which was blocked when GABA<sub>A</sub> receptor signaling in these cells was inhibited by both genetic and pharmacological means.

The observation that febrile seizures result in ectopic granule cells is important, as their abnormal position in the hilus can have an impact on excitatory hippocampal networks. Ectopic granule cells have aberrant—more complex and tortuous—dendritic paths that receive greater excitatory input than inhibitory input compared to granule cells residing in the dentate gyrus<sup>6</sup>. Axons of ectopic granule cells have also been shown to contribute to the mossy fiber innervation of CA3 as well as the abnormal sprouting of fibers into the dentate gyrus in epileptic rats<sup>7</sup>. As the output of granule cells is excitatory, excessive and

aberrant innervation of both the dentate gyrus and CA3 neurons would be predicted to render the system hyperexcitable. This is exactly what Koyama et al.<sup>5</sup> found; after EFSs, rats had hyperexcitable temporal lobes. This finding corresponds nicely with the observation that ectopic placement of granule cells in humans who had temporal lobe resections for epilepsy was found to be most common in those individuals who had febrile seizures as children<sup>8</sup>. Thus, understanding how ectopic placement of granule cells occurs could lead to crucial advances in prevention of epilepsy associated with prolonged febrile seizures.

The authors provide a compelling mechanism by which EFSs result in ectopic granule cells<sup>5</sup>. Migrating dentate granule cells have inhibitory and excitatory receptors that are used to stimulate migration. Normally, neonatal-generated granule cells follow a stereotypical maturation process, receiving tonic GABA activation before glutamate-mediated synaptic excitatory inputs develop. In developing neurons, this GABA-mediated signaling is excitatory (depolarizing) due to high intracellular concentrations of chloride.

Koyama et al.<sup>5</sup> showed that EFSs resulted in an upregulation of GABA<sub>A</sub> receptors, leading to increased sensitivity to GABA, increased intracellular Ca<sup>2+</sup> and a reversal of direction of granule cell migration. This reversal in direction of migration of the newly formed granule cells led to the cells residing in the hilus after EFSs<sup>5</sup>. Abnormally positioned neurons develop aberrant connections with other cells, and those abnormally positioned in the hilus would respond to local guidance molecules and electrical signaling, which would direct dendrite and axonal growth. Blocking GABA<sub>A</sub> receptors or voltage-sensitive Ca<sup>2+</sup> channels with bicuculline and nimodipine, respectively, prevented the ectopic localization of granule cells<sup>5</sup>, indicating that excessive GABA excitation with resulting

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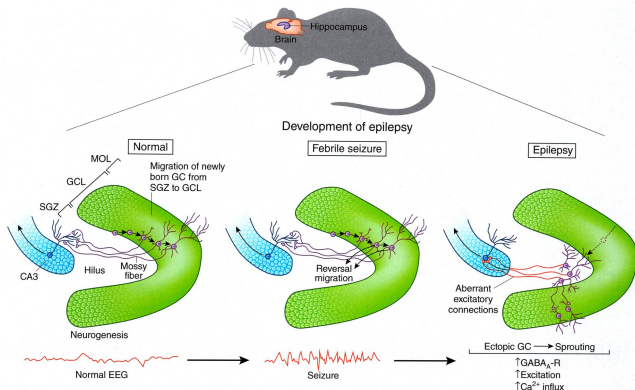


**Figure 1** Effect of experimental febrile seizures on dentate granule cell (GC) migration and epilepsy. Neurogenesis occurs in the dentate gyrus postnatally. The cells are generated in the subgranular zone (SGZ) and then migrate into the dentate granular cell layer (GCL). Dentate granule cells send their axons, called mossy fibers, to cells in CA3. After a febrile seizure, there is an increase in GABA<sub>A</sub> receptors (GABA<sub>A</sub>-Rs), leading to enhanced excitation of the migrating cells. This excessive GABA input to the migrating granule cells leads to an efflux of chloride, resulting in depolarization. With depolarization there is an influx of Ca<sup>2+</sup>, which causes a reversal in the direction of migration of granule cells, resulting in ectopic placement of the cells in the hilus. These ectopic granule cells form aberrant and functional excitatory connections with CA3 and the dentate GCL, leading to enhanced hippocampal excitability and epilepsy development. MOL, molecular layer.

increased Ca<sup>2+</sup> is responsible for the abnormal position of the granule cells.

These ectopic granule cells also rendered the hippocampus excitable<sup>2</sup>. Recordings of local electroencephalographic activity from the hippocampus showed that EFSs induced high-frequency oscillations, which appear in epileptic tissue and occur as a result of action potential bursts of synchronously discharging neurons<sup>9</sup>. Koyama *et al.*<sup>5</sup> found a positive correlation between the number of ectopic granule cells and the average duration of hippocampal high-frequency oscillations. When pilocarpine, a nonselective muscarinic receptor agonist, was used to induce seizures in the adult rats, there was activation of the ectopic granule cells, indicating these abnormally positioned cells were functionally incorporated into the network during the seizure. Thus, it seems that the ectopic granule cells have a crucial role in the development of epilepsy after EFSs. However, a word of caution is necessary—whereas it has been shown by other authors that spontaneous seizures arise from the hippocampus after EFSs<sup>3</sup>, Koyama *et al.*<sup>5</sup> did not study the origin of the seizures in their rats.

If ectopic granule cells are caused by excessive excitatory GABA, could this process be

reversed by reducing the depolarizing action of GABA in these migrating cells? The answer is affirmative. The authors used bumetanide, a selective inhibitor of the chloride transporter NKCC1, to reduce intracellular chloride concentrations. As a result, when the granule cells were activated by GABA there was an influx of chloride that caused granule cell hyperpolarization, resulting in inhibition rather than excitation. Koyama *et al.*<sup>5</sup> showed that bumetanide after EFSs eliminated the ectopic granule cells and prevented epilepsy. The authors provide a plausible explanation for why TLE occurs after febrile seizures and how treatment can prevent epilepsy from occurring—seizures increase ambient GABA produced by interneurons, causing GABA-induced upregulation of GABA<sub>A</sub> receptors on newly born granule cells, which leads to depolarization of neurons, increased Ca<sup>2+</sup> influx and a reversal of direction of migration, all of which can be blocked by reducing intracellular chloride (Fig. 1).

These results raise the question of whether children with prolonged febrile seizures should be treated immediately with bumetanide. Although this is an attractive option, there are problems with drawing this conclusion.

The authors found that repeated doses of the antiepileptic drug phenobarbital, a commonly used antiepileptic drug in both children and adults, also results in ectopic granule cells in rats, presumably through activation of GABA<sub>A</sub> receptors. However, unlike the rats with febrile seizures, these rats did not develop epilepsy. Even though ectopic granule cells may be important in the generation of epilepsy after EFSs, the fact that rats with ectopic granule cells caused by phenobarbital did not develop epilepsy indicates that other factors must be involved in the pathophysiology of epilepsy after EFSs.

Febrile seizures, like other types of seizures, induce a myriad of molecular and functional changes that might provide the mechanisms for seizure-evoked hippocampal hyperexcitability<sup>10</sup>. Focusing on one mechanism is unlikely to provide a definitive cause of the seizures. EFSs lead to altered calcium signaling in hippocampal neurons resulting in changes of gene expression<sup>11</sup>, such as genes encoding ion channels that govern the properties of H current (which contributes to the maintenance of the neuronal membrane potential) and are ideally suited for the fine-tuning of intrinsic excitability. EFS have been shown to change H current in a manner

that results in increased neuronal excitability in the hippocampus<sup>12</sup>. In addition, interleukin-1 $\beta$  signaling has been found to be crucial in fever-induced hippocampal hyperexcitability, suggesting that inflammation may contribute to subsequent epilepsy after EFSs<sup>13</sup>.

Finally, if phenobarbital results in ectopic granule cells, should its use be avoided in the treatment of seizures in infants? In addition to the finding by Koyama *et al.*<sup>5</sup> that phenobarbital causes ectopic granule cells, there is evidence that phenobarbital causes apoptosis in the developing brain of rats<sup>14</sup> and cognitive impairment after early-life prolonged seizures<sup>15</sup>. In the case of children with febrile seizures, most clinicians no longer use prophylactic phenobarbital, given there is no evidence to support the idea that prolonged therapy with anticonvulsants prevents the development of epilepsy. Chronic treatment

with phenobarbital after febrile seizures in children has been shown to hinder development of language and verbal skills acquired during the period of drug treatment, although phenobarbital had no long-term effects on intelligence quotient<sup>16</sup>. However, phenobarbital and other antiepileptic drugs that target GABA<sub>A</sub> receptors, such as the benzodiazepines, are still important in the acute management of seizures at all ages, including febrile status epilepticus, and there is no evidence in animals or humans that these drugs cause or exacerbate epilepsy. Until we find effective and safe alternatives, abandoning phenobarbital totally in children is not warranted.

**COMPETING FINANCIAL INTERESTS**  
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## Fetuin-A: the missing link in lipid-induced inflammation

Jan Heinrichsdorff & Jerrold M Olefsky

**The liver secretory protein fetuin-A (FetA) is now shown to act as an adaptor protein between free fatty acids (FFAs) and Toll-like receptor 4 (TLR4), providing the missing link between FFAs and chronic low-grade inflammation that impairs insulin sensitivity (pages 1279–1285).**

Insulin resistance is a major defect in the great majority of individuals with type 2 diabetes mellitus. Obesity is the most common cause of insulin resistance in humans, and the pervasiveness of obesity in Western populations is the predominant driver of the increasing prevalence of type 2 diabetes. Chronic low-grade inflammation in insulin target tissues is a major contributor to insulin resistance, and this reflects the interaction of proinflammatory immune cells, such as macrophages and lymphocytes, with insulin target cells, predominantly in adipose tissue and liver<sup>1</sup>. Obesity-associated inflammation is mediated, at least in part, by the effects of saturated fatty acids (SFAs) that stimulate intracellular proinflammatory pathways in a TLR4-dependent manner. Although the subject of intense study, the mechanisms connecting SFAs to TLR4 have been controversial. In this issue of *Nature Medicine*, Pal *et al.*<sup>2</sup> shed

new light on this subject by demonstrating that FetA, a liver-derived circulating glycoprotein, serves as an adaptor protein that directly links SFAs to TLR4 activation.

It has been known for some time that SFAs can stimulate inflammatory pathways in a variety of cell types, leading to decreased insulin sensitivity, and that these effects are mostly dependent on TLR4 (refs. 3,4). But exactly how SFAs accomplish this has remained the subject of debate. Some studies have postulated that SFAs can act as direct ligands for TLR4, similar to the effects of the known TLR4 ligand lipopolysaccharide (LPS). However, more recent evidence shows that direct interactions between SFAs and TLR4 are unlikely<sup>5,6</sup>. Various models have been proposed to explain this paradox. As an example of one model, SFAs may affect downstream signaling pathways by membrane reorganization. SFAs reduce membrane fluidity, promoting an accumulation of TLR4 receptors in specialized membrane domains called lipid rafts. Within the lipid rafts TLR4 undergoes homodimerization, leading to proinflammatory signaling in a reactive oxygen species-dependent manner<sup>7</sup>. SFAs can also mediate redistribution of the tyrosine kinase c-Src

into lipid rafts, and activated Src leads to stimulation of c-Jun N-terminal kinase. This study confirmed the ability of SFAs to alter the distribution of signaling molecules in cell membranes<sup>7</sup>, whereas unsaturated fatty acids such as docosahexaenoic acid (DHA) promote membrane fluidity and exert opposite effects to SFAs<sup>8</sup>. Furthermore, as SFAs exert effects on ceramide metabolism, an alternative model is that ceramide metabolism could link SFAs and TLR4. Ceramide is a lipid molecule that can cause insulin resistance, and SFAs are substrates for ceramide biosynthesis. SFAs can also stimulate increased expression of the enzymes involved in ceramide biosynthesis in a TLR4- and inhibitor of nuclear factor- $\kappa$ B kinase- $\beta$ -dependent manner. Ceramide is not essential for TLR4-dependent induction of inflammatory cytokines but is necessary for TLR4-induced insulin resistance<sup>9</sup>.

The new studies by Pal *et al.*<sup>2</sup> go a long way toward resolving how SFAs can activate TLR4 signaling despite their inability to bind TLR4 itself. The authors showed that there are elevated concentrations of FetA in the serum of obese humans and mice with diabetes when compared to healthy humans or wild-type mice and that this is accompanied by

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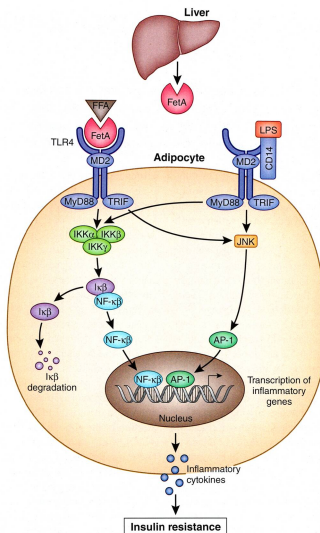


**Figure 1** FetA functions as an adaptor between FFAs and TLR4 signaling in lipid-induced inflammation. Pal *et al.*<sup>2</sup> now show the FetA, a glycoprotein produced by the liver, could provide the elusive link between FFAs and the TLR4 pathway. FFAs and LPS can both stimulate TLR4 signaling; LPS directly binds the TLR4-CD14 complex, whereas FFAs bind FetA, which then binds TLR4. TLR4 signaling leads to the activation of the transcription factors nuclear factor- $\kappa$ B (NF- $\kappa$ B) and AP-1, which can then upregulate the transcription of inflammatory genes, resulting in the production of inflammatory cytokines that can lead to insulin resistance. IKK, inhibitor of NF- $\kappa$ B kinase; I $\kappa$ B, inhibitor of  $\kappa$ B; MD2, myeloid differentiation protein-2; TRIF, TIR domain-containing adapter-inducing interferon- $\beta$ ; MyD88, myeloid differentiation factor 88.

elevated inflammatory cytokine expression. Knockdown of Tlr4 or FetA in obese insulin-resistant mice dramatically improved glucose homeostasis as a result of reduced activation of Tlr4-mediated proinflammatory signaling cascades. Most convincingly, *in vivo* SFA infusion led to the expected state of insulin resistance in control mice, but FetA-knockdown mice were protected from these effects. The authors' findings that FetA knockdown improved glucose homeostasis to a similar degree as Tlr4 knockdown and that FetA was necessary for SFA-dependent inflammatory gene expression suggested an association between SFAs, FetA and Tlr4.

Indeed, in elegant and detailed molecular studies the authors found that the fatty acid palmitate directly binds FetA with high affinity and induces strong proinflammatory responses in human adipocytes and mouse macrophages<sup>2</sup> (Fig. 1). In the absence of FetA, or in the presence of a Tlr4 inhibitor, palmitate failed to initiate proinflammatory effects. Furthermore, Pal *et al.*<sup>2</sup> showed that FetA directly binds Tlr4, and they went on to identify the protein domains on Tlr4 responsible for its interaction with FetA. Thus, SFAs bind FetA, FetA binds Tlr4, and SFAs are unable to promote Tlr4-dependent proinflammatory effects in the absence of FetA. Therefore, the authors

conclude that FetA functions as an adaptor protein that connects SFAs to Tlr4 (Fig. 1).

Moving back from the molecular details of this study by Pal *et al.*<sup>2</sup> to the broad picture, the elevated serum concentrations of FetA in obese humans raise the important question of whether the concentration of circulating FetA is correlated to the degree of insulin resistance across individuals. If so, this would place FetA as a risk factor for the development of decreased insulin sensitivity and possibly even type 2 diabetes. This study raises several other important questions. In LPS-induced TLR4 signaling, CD14 acts as a co-receptor necessary for the productive interaction between LPS and TLR4 (ref. 10). Does the FetA-induced binding of SFAs to TLR4 require a co-receptor? Furthermore, does FetA knock-out block FFA-induced dimerization in lipid rafts, and is FetA necessary for FFA-mediated c-Src activation in lipid rafts? Finally, TLR4-mediated inflammation and insulin resistance are mediated by two major stimuli; LPS directly binds the Tlr4-CD14 complex, and SFAs indirectly activate Tlr4 through FetA (Fig. 1). Although knockouts of Tlr4, FetA and CD14 all lead to improvement of glucose tolerance and insulin sensitivity, future studies will be necessary to determine the quantitative contribution of each of the mechanisms to overall *in vivo* TLR4-mediated inflammation and insulin resistance, particularly in humans<sup>10,11</sup>.

Given that most anti-inflammatory approaches to prevent insulin resistance may carry the risk of unwanted inhibition of immune functions, more specific therapeutic approaches are of great interest. Here, Pal *et al.*<sup>2</sup> provide evidence that targeting the SFA-FetA-induced branch of TLR4-mediated inflammation could have beneficial effects to improve glucose homeostasis in type 2 diabetes without affecting immune function.

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# The dark side of the oxidative force in angiogenesis

Bethany A Kerr & Tatiana V Byzova

**A new mechanism regulating pathological angiogenesis has been identified that involves the activation of ataxia-telangiectasia mutated (Atm) kinase in response to reactive oxygen species. Importantly, this Atm-dependent pathway is specifically activated in pathological, but not in normal, angiogenesis, suggesting that it could be therapeutically targeted in diseases associated with pathological angiogenesis (pages 1208–1216).**

There is a duality in the process of angiogenesis: it is beneficial during wound healing and is necessary for development, but it also has a dark side that is detrimental during tumor growth and macular degeneration. Thus, disruption of pathological angiogenesis is crucial in the treatment of ocular neovascularization and cancer. Although a number of anti-angiogenic molecules, particularly vascular endothelial growth factor (VEGF) inhibitors, are available for the treatment of pathological angiogenesis, most not only suppress pathological vessel growth but also damage healthy vasculature, leading to toxicity. In addition, resistance to VEGF inhibitors can develop as a result of compensation by VEGF-independent pathways<sup>1</sup>. Thus, to prevent and treat pathological angiogenesis, there is a need to identify new proangiogenic signaling pathways that are specifically upregulated during pathological angiogenesis and can be targeted alone or in combination with VEGF.

Several signaling pathways associated with the oxidative stress response can initiate angiogenesis. Oxidative stress plays an important part in a number of diseases associated with pathological angiogenesis, including atherosclerosis, diabetes, cancer, neurodegeneration and aging. The continued study of how different oxidative stress response pathways control angiogenesis is expected to result in the development of new therapeutic targets.

One recently identified oxidative stress pathway centers on ATM, a kinase well known for its role in orchestrating responses to DNA damage<sup>2</sup>. Mutations in *ATM* are responsible for ataxia telangiectasia, an autosomal recessive hereditary disease characterized by hypersensitivity to radiation, predisposition to hematological malignancies, ataxia (lack of voluntary muscle coordination), oculocutaneous telangiectasia (dilated blood vessels), insulin-resistant diabetes and immunodeficiency<sup>3</sup>.

Cell cycle checkpoint defects and chromosomal instability caused by *ATM* mutations and the resultant DNA repair dysfunction lead to a high incidence of cancer in patients with ataxia telangiectasia<sup>4</sup>. The *ATM* gene encodes a serine-threonine protein kinase that controls DNA repair mechanisms through its effects on p53 (refs. 3,4). During the DNA damage response (DDR), the canonical pathway is activated where ATM undergoes autophosphorylation on at least three sites (Ser367, Ser1893 and Ser1981), which breaks apart the inactive, noncovalently bound ATM dimer, resulting in the nuclear translocation and activation of the ATM-induced repair pathway (Fig. 1).

Although ATM has a classic role in DDR, not all of the pathologies arising from *ATM* mutation can be explained by this mechanism; instead, some pathologies seem to be linked to excessive oxidative stress induced by reactive oxygen species (ROS). *ATM* activation was shown<sup>5</sup> to upregulate the production of antioxidants, and *ATM*-deficient cells are more susceptible to oxidative stress-inducing agents. Correspondingly, people with ataxia telangiectasia have low antioxidant,  $\alpha$ -tocopherol and retinol plasma concentrations compared with healthy individuals and thus experience constant oxidative stress<sup>6</sup>. Likewise, *Atm*-deficient mice have high concentrations of ROS and a progressive loss of redox balance<sup>7–9</sup>, and their survival can be substantially increased by treatment with antioxidants after irradiation<sup>9</sup>. Subsequent studies have demonstrated that ATM can be directly activated by oxidation, resulting in the formation of a catalytically active disulfide-linked dimer<sup>2,4,6–8</sup> (Fig. 1). These activated dimers then initiate downstream signaling to p53 and checkpoint kinase 2, among others. Accordingly, ATM activation is involved in the responses of tissues to constitutive oxidative stress; however, how ATM deficiency contributes to the deregulation of oxidative stress remained to be elucidated.

In this issue of *Nature Medicine*, Okuno *et al.*<sup>10</sup> demonstrate that ATM is activated in sprouting vasculature in response to ROS accumulation and oxidative stress during ocular neovascularization and tumorigenesis in a mouse model. They showed that, during ischemic

retinopathy, *Atm* was activated in the tip cells of neovascular tufts and colocalized with the highest levels of oxidative tension. Notably, *Atm* was not activated in normal vasculature. To examine the role of *Atm* in pathological angiogenesis, the authors induced ischemic retinopathy in *Atm*-deficient mice and found that this prevented neovascular tuft formation. This effect was due to increased endothelial cell apoptosis and was associated with increased activation of the stress-associated kinase p38. Okuno *et al.*<sup>10</sup> showed that endothelial cell-specific *Atm* deficiency also reduced neovascular tuft formation, demonstrating that this effect was dependent on endothelial cells. Notably, the neovascular tuft formation in *Atm*-deficient mice could be rescued by administration of the antioxidant *N*-acetyl cysteine (NAC), which also abrogated p38 activation. These results suggested that *Atm* activation induces an increase in antioxidants and inhibits p38 signaling in endothelial cells, thereby leading to excessive ocular neovascularization.

To determine whether the phenomenon they observed was specific to oxygen-induced retinopathy, Okuno *et al.*<sup>10</sup> employed a mouse melanoma tumor growth model. Melanoma tumors are characterized by high amounts of inflammation, ROS and angiogenesis; correspondingly, the authors found activated *Atm* in the melanoma tumor vasculature. As with ischemic retinopathy, they demonstrated that angiogenesis and tumor growth were inhibited in endothelial-specific *Atm*-deficient mice. Again, this protection from pathological angiogenesis in *Atm*-deficient mice was dependent on p38, as deletion of endothelial p38 $\alpha$  led to increased tumor volume and vascularization compared with *Atm*-deficient mice. Thus, in oxidative stress, *Atm* is activated in newly forming vessels, inhibiting p38 activation and stimulating pathological angiogenesis.

Intriguingly, the loss of *Atm*, repression of ROS generation and activation of p38 $\alpha$  were responsible for the diminished pathological angiogenesis and shifted the balance toward normal vessel formation, without the involvement of the canonical DDR pathway. In pathological angiogenesis, ROS seems to function as a double-edged sword, as excessive

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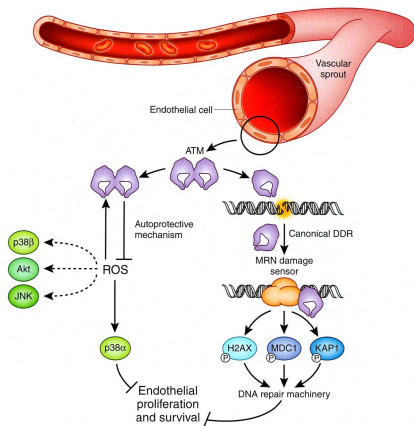


**Figure 1** Under oxidative stress, activated ATM induces angiogenesis. In endothelial cells, ATM kinase is activated, stimulating a previously unknown pathway, which has now been identified by Okuno *et al.*<sup>10</sup>. This pathway downstream of ATM induces the generation of ROS and the activation of proangiogenic pathways. Crosstalk between the canonical DDR pathway and this newly identified oxidative pathway, possibly involving the kinases p38β, Akt, JNK, H2AX, MDC1 or KAP1, may collaborate to control endothelial cell proliferation.

concentrations of ROS suppress and lower concentrations of ROS induce vascularization. Okuno *et al.*<sup>10</sup> have now discovered one proangiogenic pathway downstream of ROS, but additional pro- and antiangiogenic ROS-related pathways remain to be investigated. In addition to angiogenesis, altered concentrations of ROS can alter insulin function and glucose metabolism, stimulating the onset of diabetes, which is also prevalent in people with ataxia

telangiectasia<sup>11</sup>. ATM activation occurs downstream of ionizing radiation, DDR signaling, ROS and insulin, resulting in the activation of a variety of signaling kinases including p38, p53, Akt, hypoxia inducible factor-1α and nuclear factor-κB. Thus, modulating ATM activation could have potential applications in a variety of disease states, and an improved understanding of the different activation states (monomer versus disulfide-linked dimer), the associated

downstream signaling pathways and the cellular functions affected should provide new insights into many interrelated disease states.

Future research should focus on whether there is an overlap between the proteins targeted by ATM activation in response to DDR versus those targeted by ATM in oxidation and on how targeting ATM activation might provide therapeutic benefits for metabolic disorders, neurodegenerative disorders, pathological angiogenesis and radiation toxicity. Understanding the overlapping and divergent signaling of ATM in DDR versus oxidative stress could also bolster the use of ATM targeting in cancer treatment as, if targeting ATM was used as an adjuvant to block pathological angiogenesis, the loss of ATM signaling could affect the DDR pathway, leading to increased mutations in response to chemotherapy. Specifically, the discovery of a previously unknown, VEGF-independent proangiogenic pathway by Okuno *et al.*<sup>10</sup> opens new areas of investigation into the inhibition of pathological angiogenesis. Most importantly, the disruption of ATM signaling prevented pathological neovasculation without affecting normal vascular regeneration. Thus, ATM activation could be an ideal target for new therapies to combat pathological angiogenesis in a variety of diseases.

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## Milk modulates the microbiota

There is a growing appreciation for the role of the gut microbiota in many conditions, including obesity and inflammatory bowel disease (IBD). In a recent study, Devkota *et al.*<sup>1</sup> reveal how a dietary change can influence the microbial composition and disease pathogenesis. Feeding interleukin-10-deficient mice a diet high in saturated milk-derived fats led to the expansion of a bacterial pathobiont—a symbiotic organism that can cause pathogenesis only under certain conditions. This microbiota change increased the incidence of colitis in these genetically susceptible mice. We asked four experts to comment on the findings of this study and how they affect understanding of the complex relationship between diet, microbiota and disease.

Andrew T Gewirtz

IBD is driven by both genetic and environmental factors; two long-standing environmental suspects are diet and the large, diverse population of bacteria that inhabit the intestinal tract—the gut microbiota. That diet influences the composition of the gut microbiota and that alterations in gut microbiota composition are associated with gut inflammation suggest the possibility that these factors might, somehow, act in concert to trigger disease in susceptible hosts<sup>2,3</sup>. However, the complexity of the observed changes in microbiota composition and the lack of ability to discriminate the extent to which they were a cause or consequence of inflammation have rendered such connections to be inchoate hypotheses.

In contrast, the work of Devkota *et al.*<sup>1</sup> serves as a remarkably well-defined example of how a discrete change in diet can selectively alter gut microbiota composition and drive colitis in genetically susceptible hosts. As the dietary change, namely the presence of milk fats, caused very similar changes in the microbiota composition in susceptible and non-susceptible mice, this indicates that the microbiota alterations are not merely a consequence of colitis. In addition, the ability of the observed microbiota change—the increase in *Bifidobacterium wadsworthii*—to cause colitis in susceptible hosts in the absence of any other bacteria demonstrates its contribution in driving disease. This work is a very important advance in understanding the role of the microbiota in general and IBD in particular for two primary reasons. First, it yields a hypothesis, which can be tested in humans, that diets that alter bile acids can induce the selective outgrowth of sulfite-reducing pathobionts that might drive IBD. Second, it provides a blueprint for future mechanistic studies to investigate the complex interactions between the intestinal immune system and the microbiota in a defined and rigorous manner.

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### COMPETING FINANCIAL INTERESTS

The author declares no competing financial interests.

## Sarkis K Mazmanian

Infectious diseases and their hosts have cohabited the Earth for time immemorial<sup>4</sup>. However, recent evidence suggests a previously unrecognized option for infectious disease—microbes that have a tenuous alliance with a host that is not fit enough to eliminate the pathogen, yet not susceptible enough to be killed by the infection. Such pathogens may persist peacefully under most conditions as part of the microbiota but can cause disease under certain genetic or environmental conditions<sup>5</sup>. My group has termed such microbes ‘pathobionts’<sup>6,7</sup>.

Devkota *et al.*<sup>1</sup> now elegantly (and for the first time) demonstrate that the combination of dietary and genetic changes in mice leads to disease driven by a pathobiont of the gut microbiota. The authors show that recapitulating the high-saturated-fat diet prevalent in Western societies in mice causes the species *B. wadsworthii* to expand from a minor to a substantial component of the mouse gut microbiota. When mice genetically susceptible to colitis are fed the same diet, they not only show the same expansion of *B. wadsworthii* but also have increased incidence and severity of intestinal disease.

Is *B. wadsworthii* the cause of increased colitis or simply coincident with disease? The authors have fulfilled Koch's postulates by also culturing the pathobiont and monocolonizing genetically susceptible germ-free mice<sup>1</sup>. The organism flourishes under conditions where

## Fredrik Bäckhed

It has become evident that most genes in the human body are contained within its microorganisms (the microbiome). Studies in animals have revealed a causal relationship between the gut microbiota and several diseases. The microbiome composition is affected by the gene repertoire of the host and by ingested nutrients and other environmental cues. Thus, the microbiome constitutes a plastic part of the human metagenome (the combination of human and microbial genes), which may acquire new functions and substantially affect host physiology.

Recently, Devkota *et al.*<sup>1</sup> demonstrated that *B. wadsworthii*, a sulfite-reducing pathobiont implicated in IBD, was greatly expanded in the mouse gut upon feeding of a diet rich in milk fat, whereas polyunsaturated fatty acids did not affect *B. wadsworthii* abundance. *B. wadsworthii* expansion was associated with an increased proportion of taurine-conjugate cholic acid (TCA) in the gall bladder and probably also in the gut upon exposure to milk fat. Taurine is a rich source of sulfite, which

may explain the observed increase in *B. wadsworthii*.

The milk-fat-induced expansion of *B. wadsworthii* was associated with colitis in genetically susceptible *Il10*-deficient mice<sup>1</sup>. Milk-fat-fed wild-type mice subjected to dextran sodium sulfate (DSS) also developed colitis, demonstrating that the immune deficiency *per se* did not cause the phenotype. DSS-treated mice have an impaired mucus structure that allows larger particles and bacteria to penetrate the mucosal surface<sup>8</sup>. So, a second ‘hit’, such as impairment of the mucus layer, which would enable the bacteria to come into close contact with the epithelium, might be important for driving colitis, as *B. wadsworthii* expansion was also observed in wild-type mice who did not have gut inflammation. Mechanistically, the authors showed that *B. wadsworthii* induced a T helper type 1 (T<sub>H</sub>1) immune response in the mucosa and mesenteric lymph nodes and also caused a significant increase in production of hydrogen sulfide<sup>1</sup>, which is toxic to epithelial cells.

## Jeremy K Nicholson

taurine-conjugated bile acids are formed. Intriguingly, the diet that promotes *B. wadsworthia* expansion stimulates such bile acids, and feeding with taurine-conjugated bile acids alone enhances disease. Although bile is generally destructive to most microbes, this species has evolved to use it as a nutrient. Given that gut bacteria eat what we eat, are Western diets selecting for pathogens that promote disease?

IBDs have dramatically increased in prevalence in developed societies over the past few decades<sup>8</sup>, suggesting that the driving forces behind this increase may be environmental changes. However, environmental changes, such as changes in dietary habits, may affect a subset of the population whose genetics that make them more susceptible to colitis. Devkota *et al.*<sup>1</sup> have brought together these two variables in a mouse model to show that they may work in combination to cause increased colitis in mice. Future studies should aim to determine the mechanisms by which *B. wadsworthia* and other pathogens cause disease, thus identifying targets for new therapies for Crohn's disease, ulcerative colitis or both.

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## COMPETING FINANCIAL INTERESTS

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One important difference between mice and humans is that human bile contains high concentrations of non-sulfur-containing glycine-conjugate cholic acid and lower concentrations of TCA, which limits the clinical relevance of the present study<sup>1</sup>. That said, this paper elegantly demonstrates that not only the quantity, but also the quality, of fat is important for shaping the microbiome and that the 'wrong' diet, together with the microbiota, may lead to disease progression in susceptible hosts. Thus, these findings fur-

ther emphasize the importance of the microbiome in IBD.

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It is now recognized that the gut microbiome is a major mediating factor involved in the etiopathogenesis of many diseases, including IBD, that have become increasingly common over the last few decades<sup>10</sup>. The paper by Devkota *et al.*<sup>1</sup> elegantly illustrates the complex multicompartmental connections between host genetics, dietary composition, the microbiome and immune function in the development of colitis in mice. Specifically, the authors observed that high amounts of dietary saturated milk fat change the gut microbial assemblage and promote the growth of sulfite-reducing bacteria in *Il10<sup>-/-</sup>* (but not wild-type) mice. This is associated with increased hepatic bile acid taurine conjugation, a T<sub>H</sub>1 immune response and the development of colitis.

"These targets do not even have to be on the human side of the interaction, as the human microbial genome itself may be druggable."

Although the mouse microbiome is very different from the human microbiome in terms of species composition, there are many structural ecological parallels and similarities between them. Thus, this work provides a plausible underlying mechanism for understanding inflammatory bowel conditions in humans. The microbiota are involved in regulating a series of metabolic signaling and immune-inflammatory axes that physiologically connect the gut to many other body compartments. The 'bile acid nuclear receptor axis'<sup>11</sup>, which involves the microbial metabolism of bile acids of endogenous origin to different pharmacologically active compounds that interact with peroxisome proliferator-activated receptors, is increasingly being recognized for its importance in the modulation of gene expression and the co-regulation of major metabolic pathways, including cholesterol and steroid synthesis<sup>12</sup>. The work of Devkota *et al.*<sup>1</sup> is thus of further value, as it gives insight into the transgenomic metabolic and immune signaling processes that underpin the microbial role in the development of colitis, and this may offer new potential therapeutic targets. These targets do not even have to be on the human side of the interaction, as the human microbial genome itself may be druggable and the microbiota responds to both dietary and probiotic interventions. However, a much deeper understanding of the function of these transgenomic axes and their links to host physiology is a prerequisite for optimizing therapeutic strategies to manipulate the gut microbiota for human health. We are still some way from the required level of knowledge, but this new work<sup>1</sup> provides another stepping stone to aid understanding human disease processes in the age of the symbiotic supraorganism.

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## COMPETING FINANCIAL INTERESTS

The author declares no competing financial interests.

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# A complex microworld in the gut

Millions of healthy bacteria colonize our guts from the moment we are born. Changes in the composition and abundance of these commensals affect the entire immune system and can predispose us to a variety of diseases, including intestinal infections, inflammatory and metabolic diseases, and cancer. The gut microbiome interacts not only with the host mucosa but also with potential pathogens; understanding what interactions and pathways are crucial for maintaining homeostasis and protecting the host from harmful bacteria and diseases can open new avenues to developing gut microbiota-based therapeutic approaches. In 'Bench to Bedside', Michael R. Howitt and Wendy S. Garrett examine the importance of metabolic crosstalk between the microbiota and the host in human metabolism and the development of cardiovascular disease. This adds one more layer of complexity to understanding what contributes to this pathology and how to harness the microbiota and their metabolic pathways to prevent it. In 'Bedside to Bench', Nobuhiko Kamada, Grace Chen and Gabriel Núñez discuss how targeting interactions between commensals and bacteria causing intestinal disease can lead to effective therapies to control these infections, which currently seem to lack an adequate treatment. Unraveling how commensals help the host prevent or block colonization of these pathogens can suggest new ways to increase our armamentarium to deal with these sometimes deadly intestinal infections.

## ■ BENCH TO BEDSIDE

# Gut microbiota and cardiovascular disease connectivity

Michael R Howitt & Wendy S Garrett

The intestinal microbiota of mammals imbues their hosts with a greatly expanded repertoire of enzymes to digest and metabolize food. Microbes such as *Bacteroides* spp., which are prevalent in the gastrointestinal tracts of both mice and humans, express a suite of glycoside hydrolase enzymes that facilitate the digestion and extraction of energy from complex polysaccharides<sup>1</sup>. Mice lacking a microbiota—germ-free mice—consume 27% more food than their conventional counterparts yet have significantly less body fat; highlighting the role of the microbiota in metabolism and energy balance<sup>2</sup>. In the past decade, the expanded use of germ-free mice and improvement in various 'omics' technologies have ushered in a burgeoning understanding of how the microbiota affects human health.

The recent publications by the Human Microbiome Project and the recently completed MetaHIT project are two multi-institutional projects that have created a massive metagenomic database of the human microbiome<sup>3–6</sup>. By establishing a comprehensive assessment of the human microbiome in healthy individuals, these studies provide a valuable platform from

which future research can define changes to the microbiome as they apply to both health and disease. Metagenomic data from both animal models and patient cohorts are expanding the recognition of functions performed by the microbiota in energy-harvest and associated metabolic diseases: obesity<sup>7</sup>, metabolic syndrome<sup>8</sup> and nonalcoholic fatty liver disease<sup>9</sup>. Yet the metabolic crosstalk between the microbiota and host extends functionally far beyond energy acquisition and geographically far beyond the gut.

The products of microbial metabolism are crucial for the development and tuning of the mucosal immune system and enhancing epithelial barrier function<sup>10</sup>. However, microbial metabolism can also have a deleterious effect on the host. Microbial conversion of dietary choline to trimethylamine contributes to nonalcoholic fatty liver disease in susceptible mice<sup>11</sup>. This same metabolic pathway re-emerged in a recent study that analyzed blood plasma from a cohort of individuals undergoing noninvasive cardiac stress testing to determine metabolite signatures linked to cardiovascular disease (CVD)<sup>12</sup>. By comparing plasma metabolites from cohorts of subjects with CVD and healthy subjects, Wang *et al.*<sup>12</sup> found associations between coronary artery disease, peripheral vascular disease and history of myocardial infarction to the metabolites choline, trimethylamine *N*-oxide (TMAO) and betaine.

Metabolomic examination of human

plasma analytes and mouse feeding studies of phosphatidylcholine permitted reconstruction of the metabolic pathway by which dietary phosphatidylcholine was metabolized to choline, TMAO and betaine. Although prior work demonstrated that the gut microbiota is essential in generating TMAO<sup>13</sup>, the link between microbial conversion of choline to TMAO and an association with CVD had not been previously observed, nor had there been deep consideration of a role for dietary phospholipids in CVD pathophysiology. This study supports the notion that the gut microbiota has relevance for human health and disease beyond the gastrointestinal tract<sup>12</sup>.

Whereas the phosphatidylcholine-derived CVD biomarkers observed by Wang *et al.*<sup>12</sup> in humans were both interesting and robust, a particular strength of the study came from the use of rodent models. Oral feeding of phosphatidylcholine, rather than parenteral delivery, was necessary to generate the metabolite TMAO, suggesting that a necessary step in this biochemical pathway was performed only within the gut. In both conventional mice treated with antibiotics to perturb the microbiota and germ-free mice, generation of TMAO and specifically of its precursor, TMA, was dependent on the gut microbiota (Fig. 1).

Furthermore, in atherosclerosis-prone (*Apoe*<sup>-/-</sup>) mice the phosphatidylcholine metabolites exacerbated disease in a microbiota-dependent fashion<sup>12</sup>. In humans, hepatic

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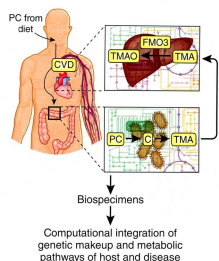


**Figure 1** Gut microbiota metabolism of phosphatidylcholine and CVD. Intestinal microbes metabolize dietary phosphatidylcholine (PC), generating trimethylamine (TMA). In the liver, trimethylamine is converted to TMAO by flavin-containing monooxygenases (FMOs). A metabolomic study has shown an association between CVD and the metabolites TMAO, choline (C) and betaine. This study supports the importance of future investigation of the microbiota in human biospecimens and the need for computational pipelines that can integrate data on human and microbial genetics to shed light on the metabolic pathways of the host and the microbiota in health and disease.

flavin-containing monooxygenase 3 (FMO3) is necessary for TMAO production; accordingly, the authors analyzed liver biopsy samples and found a positive correlation between FMO3 expression and plasma TMAO concentration. Atherosclerosis-prone mice also showed a correlation between FMO3 expression and CVD. In an effort to link TMAO levels more directly to atherosclerosis, the authors showed that elevated plasma TMAO concentrations were associated with upregulated macrophage scavenger receptors and increased foam cell numbers in mice<sup>12</sup>. As macrophages and foam cells are found in human atherosclerotic lesions, it will be important to consider the metabolic inputs that shape macrophage function and how microbial metabolites affect the endothelium. Collectively, the results of this study defined interconnections between host and microbial chemistry in metabolizing dietary substances and the contribution of such metabolites to CVD.

Large cohort studies, such as the Framingham Heart Study, have been instrumental in defining the principal risk factors for stroke and myocardial infarction, including serum lipid profiles and hypertension.

Diet-related obesity in turn can influence both of these risk factors. Whereas there are simple and effective diagnostic and management tools for obesity and hypertension (scales and sphygmomanometers), new observations that microbiome-based profiles or serum metabolites can also identify these health problems suggest opportunities for microbiome-based insights into disease mechanisms and hope for predicting, preventing and treating diverse disorders. The microbiome clearly shapes the generation of dietary metabolites, as do host genetics and host enzymatic pathways<sup>14</sup>. Human metabolism can therefore be seen as a collection of both microbial and human metabolic capabilities. Clinical cohorts with lifestyle, medication and medical history metadata and linked biorepositories of serum, urine and fecal samples and tissues are a treasure trove that will afford new insight into how the microbial world acts to influence human physiology and disease.

For those interested in beginning to analyze the microbiome in clinical samples and discerning whether there are bacterial taxonomic-based biomarkers associated with specific health and disease states, 16S rRNA gene surveys are an affordable and accessible approach. However, moving from microbial community structure data to bacterially generated metabolites requires not only a vast number of well-annotated genomes but also computational platforms that can link 16S rRNA gene survey data to bacterial metabolic pathways. The reverse, moving from metabolites and enzymatic pathways to specific bacterial genes and species, is also far from a trivial task, involving complex metabolic profiling and functional genomics. Wang *et al.*<sup>12</sup> have provided a seminal contribution in identifying a host-microbiota metabolic node contributing to CVD; however, the microbial pathways and species that contribute to phosphatidylcholine metabolite production remain to be determined.

Omics technologies will continue to offer insight into how human and microbial genetics and lifestyle contribute to CVD. There are an increasing number of publications reporting patterns in the microbiota that can distinguish persons with a disease from healthy individuals. Cognizant of inter-individual genetic and microbiota variation, studies examining afflicted individuals over periods of health and disease will prove essential for not only leveraging the microbiota as biomarker but also providing a window into disease causality of gut microbes and their metabolites. However, substantial

investments in biospecimen processing, analytical methods and computational infrastructure are still needed to fully implement the microbiota as a biomarker, diagnostic or therapeutic armamentarium. The study by Wang *et al.*<sup>12</sup> supports that this investment is worthwhile and will be an important test case going forward.

Will investigators be able to define specific inhibitors to inhibit TMAO or trimethylamine generation and prevent CVD or treat established disease? Such work will involve collaboration between chemists, microbiome investigators and preclinical and clinical investigators. An interesting corollary to these studies is determining how existing drugs, such as aspirins or statins that target modifiable CVD risk factors, affect the microbiota<sup>15</sup> and are acted on by the microbiota<sup>16</sup>. Reconstructing both host and microbial metabolic networks is a primary challenge, and determining their interconnections will be a complex task. Undoubtedly, targeting pathways with host and microbial enzymatic contributions will unveil feedback and feed-forward regulatory mechanisms that may complicate development of therapeutic interventions. The generation of the requisite computational pipelines and the optimization of model-systems to unravel host-microbiota interactions promise to be enthralling endeavors. The microbiota has become a rediscovered old entity that offers a new frontier for defining metrics for evaluating health status and, hopefully, fruitful therapeutic avenues for mitigating risk of disease.

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## ■ BEDSIDE TO BENCH

# Harnessing pathogen-commensal relations

Nobuhiko Kamada, Grace Chen & Gabriel Núñez

Human infection by diarrheagenic *Escherichia coli* strains, including enterohemorrhagic (EPEC), enteropathogenic (EPEC), enteroaggregative (EAEC), enteroinvasive (EIEC) and enterotoxigenic (ETEC) *E. coli*, cause substantial morbidity and mortality worldwide each year<sup>1</sup>. The most effective way to protect from infection is through primary prevention by improving sanitation and personal hygiene to limit the transmission of bacteria through fecal contamination of food and water supplies. Other measures include eradication of sources of pathogenic bacteria, such as infected cattle, a major source of infection in the developing world. Unfortunately, effective therapies to thwart these water- and food-borne intestinal diseases are lacking. A major factor hampering the development of curative therapies is the poor understanding of how microbial and host factors regulate the colonization and eradication of these deadly bacterial pathogens.

Unlike the harmless commensal *E. coli* that reside in the human intestine, pathogenic *E. coli* produces virulence factors and toxins that are crucial for its ability to divide and induce disease. For example, strains of EPEC and EAEC can produce Shiga toxin that contributes to the development of hemolytic uremic syndrome, which is characterized by the triad of acute renal failure, hemolytic anemia and thrombocytopenia. Likewise, ETEC, the most common cause of traveler's diarrhea, produces one or more enterotoxins and fimbriae necessary for attachment to the host intestinal epithelium<sup>1</sup>. In contrast to EAEC and ETEC, the genomes of EPEC and EHEC harbor the locus of enterocyte effacement (LEE), which is crucial for pathogen colonization and disease pathology<sup>2–3</sup>. This locus contains a cluster of virulence genes that are crucial for pathogen colonization and include those encoding the type III secretion system (T3SS), a molecular needle-like structure that allows injection of bacterial effector proteins into the host cell cytosol, of effector proteins injected via the T3SS, and regulators of epithelial attachment, such as Tir and intimin<sup>3</sup>.

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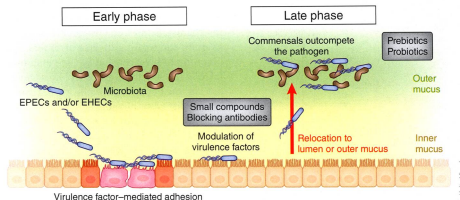


**Figure 1** Potential therapeutic strategies to treat pathogenic *E. coli* infection that target pathogen virulence factors and the gut microbiota. At the early phase of infection, EPEC and EHEC LEE virulence factors that mediate localization of the pathogen at or near the intestinal epithelium allow its replication in the presence of the microbiota. At the late phase of infection, LEE virulence is downregulated, which leads to relocation of the pathogen to the intestinal lumen and outer mucus layer where it is outcompeted by metabolically related commensals. Targeting LEE virulence (using small-molecule compounds or blocking antibodies) and/or populations of commensals (using prebiotics or probiotics) may be helpful in treating infection by promoting commensal-driven pathogen eradication.

A feature of pathogenic *E. coli* strains is their genetic heterogeneity even among bacteria that are classified within the same group. A clear example of this is the recent outbreak of enteritis and hemolytic uremic syndrome caused by an enterovirulent *E. coli* strain in Germany in 2011, which affected almost 4,000 individuals and caused more than 50 deaths<sup>4</sup>. Genomic analysis revealed that the pathogenic *E. coli* strain (serotype O104:H4) that infected the German patients was genetically most similar to the EAEC strain 55989, and therefore it was classified as an EAEC. Specifically, like typical EAEC strains, it lacked the LEE but contained virulence genes coordinately regulated by the AggR transcription activator, including those encoding aggregative adherence fimbria (*aggA-aggD*) for attachment to the intestinal epithelium and dispersin (*aap*), which facilitates bacterial dispersal along the intestinal mucosa surface<sup>5</sup>. Despite its genetic similarity to EAEC, the German strain was unusual in that it also carried genetic elements associated typically with EHEC, namely the *Stx2* gene encoding Shiga toxin 2 and antibiotic resistance genes, which may partly explain its ability to cause severe illness and hemolytic uremic syndrome<sup>6,7</sup>. However, the role of these virulence genes in eliciting morbidity and lethality requires further investigation. Knowing what virulence factors are present and important for

pathogenesis may ultimately define new targets for therapeutic intervention.

This outbreak highlights the importance of collaborative efforts to identify and characterize the causative agent as well as the vehicle of transmission and origin of infection. Unfortunately, once an individual is infected, there is no effective treatment that targets the great majority of enterovirulent *E. coli* strains. Although antibiotics are commonly used, they have largely been ineffective and are also associated with an increased risk of hemolytic uremic syndrome in both children and adults<sup>8</sup>. There are several reasons that may explain the limited utility of antibiotics. First, pathogenic *E. coli* is inherently antibiotic resistant, as was the case with the German outbreak strain that expressed an extended-spectrum  $\beta$ -lactamase<sup>9,7</sup>. Second, certain antibiotics can trigger the production of Shiga toxin, which is associated with the development of hemolytic uremic syndrome<sup>9</sup>. Finally, antibiotics also eliminate populations of commensals that are necessary for the clearance of pathogenic *E. coli* during infection<sup>10,11</sup>. Thus, there is a need for the development of new therapeutic strategies to manage infections with enterovirulent *E. coli* strains.

An alternative strategy to antibiotics is to harness the gut microbiota. Recent studies have revealed that the gut microbiota plays a crucial part in the eradication of *Citrobacter rodent-*

*tium*, a natural mouse pathogen that models infection by EPEC and EHEC in humans<sup>11</sup>. During the early phase of infection, LEE virulence genes are induced, allowing the pathogen to attach to the epithelium lining and multiply in the presence of the intestinal microbiota<sup>11</sup>. During the late phase of infection, when LEE virulence is downregulated and the pathogen moves to the intestinal lumen, metabolically related commensals are involved in pathogen clearance by outcompeting pathogens for limited nutrients (Fig. 1). These studies suggest that targeting LEE virulence at the early phase of infection may be an effective approach to treating EPEC and EHEC infections (Fig. 1). The host or microbial factors that cause downregulation of LEE virulence remain unknown, and elucidation of the mechanism that regulates LEE virulence in the intestine during infection could be key in identifying new targets for bacterial eradication.

How commensals mediate colonization resistance against enteric pathogens remains poorly understood. In the *C. rodentium* model, the capacity to forage carbohydrates determines, at least in part, the ability of gut commensals to outcompete the pathogen for energy resources<sup>11</sup>. Thus, the oral administration of certain beneficial commensals (probiotics), or treatment with dietary ingredients that boost the growth of natural 'competitors' (prebiotics), may prove to be effective treatment approaches (Fig. 1). This approach may be effective in eradicating not only enterovirulent

*E. coli* infection but also other intestinal infections, such as *Clostridium difficile* infection (CDI), which is a leading cause of healthcare-associated infectious diarrhea and colitis<sup>12</sup>. Infection with *C. difficile*, an obligate anaerobic, Gram-positive, spore-forming bacterium, is associated with the use of broad-spectrum antibiotics in hospitalized patients and is presumed to be due to the removal of protective commensal bacteria by antibiotics. *C. difficile* is present in the intestine of ~5% of adult healthy individuals, but it is not a common member of the gut microbiota. Thus, disruption of the indigenous gut microbiota, which is important in halting the growth of *C. difficile* by antibiotics, may make the intestine vulnerable to colonization by exogenous *C. difficile* spores or pathogen growth in previously colonized individuals.

Although treatment with certain antibiotics such as metronidazole or vancomycin is usually effective in treating CDI, intestinal microbiota transplantation is used to manage recurrent infection that is refractory to antibiotic therapy<sup>12,13</sup>. This transplantation approach involves infusing microorganisms from a healthy donor stool into the intestine of a patient to restore the normal microbiota. Although intestinal microbiota transplantation can be effective in treating recurrent CDI, the variability of the donor commensal populations may hamper its clinical use. Thus, identification and characterization of the gut commensals that mediate protection against

*C. difficile* may lead to the use of single species or defined cocktails of commensals to treat CDI. Furthermore, understanding of the metabolic pathways that are used by commensals to outcompete pathogens may lead to the genetic engineering of commensal species with increased capacity to limit pathogen colonization. Such approaches could improve our armamentarium to treat intestinal infections including those caused by *C. difficile* and enterovirulent *E. coli*.

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The authors declare no competing financial interests.

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## ■ NEUROSCIENCE

## It's all in the details

A rare variant of Epac2 that is found in some individuals with autism preferentially affects the basal over the apical dendrites of cortical neurons (PLoS Biol. 10, e1001350). The specificity of this effect suggests that the mutations found in people with autism may result in relatively subtle effects on brain architecture.

Epac2 is a brain-enriched guanine nucleotide exchange factor that regulates the function of the small GTPases Rap and Ras. Deepak Srivastava *et al.* found that knocking down Epac2 in cortical neurons *in vitro* reduced the size and complexity of their basal dendritic arbors. Overexpressing an autism-associated Epac2-coding variant had a similar effect. Mechanistically, this mutation disrupted the interaction between Epac2 and Ras. Indeed, inhibiting Ras also impaired basal-dendrite architecture.

As the number of identified rare coding variants associated with autism increases, it will be of interest to see whether they also result in phenotypes with an equal degree of specificity within a single cell. It will also be important to establish whether the autism-associated Epac2 mutation has a similar effect on neurons from other brain regions and whether there is a developmental window during which the impairment on basal-dendrite architecture can be reversed.—JCL

## ■ NEUROSCIENCE

## Restricted astrocytes

Astrocytes are a type of glial cell and are essential for the maintenance of many neurological functions. Now, a new study (Science doi:10.1126/science.1222381) reveals that astrocytes are localized to specific domains in the central nervous system, with potential consequences for understanding region-specific brain functions.

Using reporter fate mapping in mice, Hui-Hsin Tsai *et al.* found that astrocytes migrate radially from the ventricular zone, forming distinct domains that are determined by their embryonic sites of origin in this zone. The astrocytes did not undergo secondary tangential migration later in development, in adulthood or after injury, suggesting that the restricted astrocyte domains remain stable.

The authors then investigated how astrocyte distribution influences interactions with neighboring neurons by selectively removing astrocytes from part of the ventral ventricular zone (the motor neuron progenitor domain). This

## Virology

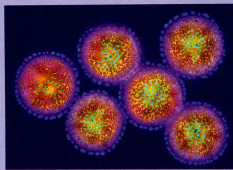
## 'Influenzing' the host response

A recent study identifies a new protein produced by the influenza A virus (IAV) that can modulate the host response, thus providing insights into pathogenesis by this virus (Science 337, 199–204).

Segment 3 of the IAV genome encodes a single mRNA that encodes a subunit of the viral RNA-dependent RNA polymerase complex (PA), and Jagger *et al.* found that this viral segment contains an additional open reading frame (X-ORF) that can yield a different protein, PA-X, through ribosomal frameshifting. They showed that PA-X is composed of the endonuclease domain of PA and a C-terminal domain encoded by X-ORF and functions to repress the expression of cellular genes.

Jagger *et al.* then compared the pathology in mice caused by a reconstructed 1918 pandemic IAV that expressed PA-X with mutant IAVs that did not express PA-X. They found that the mutated viruses caused more severe disease than the wild-type 1918 pandemic virus, suggesting that PA-X reduces the pathogenicity of IAV. Moreover, infection with the mutated IAVs was associated with changes in host gene expression, including increased expression of genes involved in inflammation, apoptosis and T cell signaling compared with the wild-type IAV, as well as a reduced ability to induce host-cell shutoff.

This study contributes to our understanding of the roles of the virus and the host immune response in IAV pathogenesis, suggesting that immunopathology is a key aspect of IAV-mediated disease.—MS



James Cavillier / Photo Researchers, Inc.

depletion altered the numbers of certain types of synapses, and astrocytes from adjacent regions could not migrate into the depleted area to rescue the synaptic dysfunction.

Thus, these results indicate that regional astrocyte allocation might be important for the development and maintenance of localized brain functions. The findings could also have implications for understanding brain regeneration, as they show that there are limitations in the astrocyte response to injury.—MS

## ■ CANCER

## Stromal protection from therapy

Chemotherapy is the most common therapeutic option for metastatic breast cancer, but resistance readily arises. The link between metastasis and chemoresistance remains poorly understood. A recent report (Cell 150, 165–178) uncovers a signaling network linking metastatic and chemoresistant behavior and expands our understanding of the emerging role of the tumor microenvironment in controlling therapy responses.

Studying two related proteins previously associated with metastasis, CXCL1 and CXCL2 (CXCL1/2), Acharyya *et al.* find that they play a prometastatic part in cancer through their

paracrine action on myeloid cells. Moreover, CXCL1/2-recruited myeloid cells can secrete factors that promote not only growth and metastasis but also the survival of cancer cells after chemotherapy.

Interestingly, the production of CXCL1/2 is upregulated in tumor cells treated with chemotherapy drugs. This stems from the proinflammatory effect of such therapies on normal tissues, leading stromal components to release cytokines that can upregulate the expression of CXCL1/2.

The authors find evidence that this signaling loop operates in humans with metastatic breast cancer and suggest that it may be a future target to prevent microenvironmental components from creating a protective milieu that allows the acquisition of therapy resistance.—VA

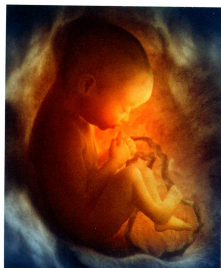
## ■ REPRODUCTIVE BIOLOGY

## Bringing up baby

Whereas most expectant mothers love their gestating babies, their immune systems do not, instead seeing them as foreign bodies because of the paternal alloantigens they harbor. A new report (Cell 150, 29–38) gives further insight into how maternal-fetal tolerance is maintained.

Peripherally-generated regulatory T (pT<sub>reg</sub>) cells are believed to restrain immune responses





Jellyfish Pictures / Photo Researchers, Inc.

to nonself antigens, such as allergens, commensal microbiota and food, whereas thymically generated regulatory T ( $T_{reg}$ ) cells are thought to be responsible for tolerance to self antigens during development. Interestingly,  $pT_{reg}$  cells are known to harbor an enhancer element, conserved noncoding sequence 1 (CNS1), in the promoter of *Foxp3* (encoding a transcription factor required for  $T_{reg}$  cell function) that allows for the development of  $pT_{reg}$  cells but is dispensable for the generation of  $tT_{reg}$  cells. Samstein *et al.* show that CNS1 is unique to placental mammals and that it is required for the generation of  $pT_{reg}$  cells specific for fetal alloantigens during pregnancy in mice. They also show that the absence of CNS1 results in more fetal absorption by pregnant dams when mated to allogeneic males but has no effect when mutant females are mated to syngeneic males.

These results suggest that  $pT_{reg}$  cells are a crucial component of maternal-fetal tolerance and that the system arose during mammalian evolution.—*RL*

## ■ IMMUNITY

### Gut bugs alter antiviral immunity

At mucosal surfaces, commensal microbes can promote the development of pathogenic and regulatory adaptive immune cells that help contain pathogens and prevent infection. Whether commensal bacteria can regulate systemic immunity to viruses, however, remains unclear.

Two research groups now report that depletion of the gut microflora impairs the clearance of a viral infection. Andreas Diefenbach and his colleagues (*Immunity* doi:10.1016/j.immuni.2012.05.020) found that natural killer cell activity was impaired in germ-free mice. These splenic natural killer cells failed

to receive priming signals from dendritic cells, which in response to viral infection, normally produce type I interferons (IFN-I). The serum concentrations of IFN-I were reduced in virally infected germ-free mice, and IFN-I injection normalized natural killer cell responses.

David Artis (*Immunity* doi:10.1016/j.immuni.2012.04.011) and his colleagues report that virus-specific adaptive immune responses are impaired in antibiotic-treated mice. The expression of genes that regulate antiviral immunity was decreased in macrophages isolated from the gut of antibiotic-treated mice. These macrophages responded weakly to IFN stimulation and were impaired in their ability to control viral infection.

Taken together, these studies reveal distinct ways in which commensal bacteria can influence innate immune activation and antiviral immunity.—*KDS*

## ■ CARDIOVASCULAR DISEASES

### STAMPing down inflammation

Macrophage activation, which contributes to vascular inflammation, is a central feature of atherosclerotic plaque initiation and development. Gökhan Hotamisligil and his colleagues now identify a new anti-inflammatory mechanism in macrophages that protects against atherosclerosis in mice (*Cell Metab.* 16, 81–89).

These authors previously showed that the transmembrane protein Stamp2 is needed for normal metabolic homeostasis in mice, including in adipose tissue and liver. In their new work, they show that Stamp2, which was previously characterized as having reductase activity, lowers the amounts of NADPH in macrophages. The increased amounts of NADPH in Stamp2-deficient macrophages led to a proinflammatory phenotype with an increased expression of a host of inflammatory cytokines. Stamp2 deficiency also decreased cholesterol efflux and enhanced foam cell formation. To connect these findings to atherosclerosis, the researchers showed that Stamp2 is expressed in macrophages of atherosclerotic plaques in both humans and mice and that Stamp2 deficiency in mice promoted vascular inflammation and increased the size of atherosclerotic lesions. Stamp2 deficiency only in bone marrow cells also resulted in increased atherosclerotic lesion size, consistent with the idea that Stamp2 acts in macrophages to restrain inflammation and atherosclerosis.—*MB*

Written by Victoria Aranda, Michael Basson, Kevin Da Silva, Randy Levinson, Juan Carlos López and Meera Swami

## New from NPG

### Non-invasive prenatal measurement of the fetal genome

Fan, H.C. *et al.* *Nature* doi:10.1038/nature11251 (4 July).

The authors describe the complete sequencing of the fetal genome using a non-invasive method. This involves a molecular counting method to analyze the parental haplotypes in maternal plasma, allowing the fetal genome to be determined from this information.

### NLRP6 negatively regulates innate immunity and host defence against bacterial pathogens

Anand, P.K. *et al.* *Nature* doi:10.1038/nature11250 (1 July).

Mice lacking the NOD-like receptor *Nlrp6*, which is involved in inflammasome signaling, are resistant to several bacterial pathogens. Infected *Nlrp6*-deficient cells produced more inflammatory cytokines, suggesting that NLRP6 downregulates inflammatory signaling and impede the systemic clearance of bacterial pathogens.

### De novo germline and postzygotic mutations in *AKT3*, *PIK3R2* and *PIK3CA* cause a spectrum of related megalencephaly syndromes

Rivière, J.-B. *et al.* *Nat. Genet.* doi:10.1038/ng.2327 (24 June).

### De novo somatic mutations in components of the PI3K-AKT3-mTOR pathway cause hemimegalencephaly

Lee, J.H. *et al.* *Nat. Genet.* doi:10.1038/ng.2329 (24 June).

Two recent exome sequencing studies provide insights into the genetic basis of megalencephaly syndromes, sporadic overgrowth disorders associated with an enlarged brain size. Both studies identify *de novo* mutations in genes encoding components of the PI3K-AKT3-mTOR pathway, suggesting this pathway is important in driving tissue overgrowth in these conditions.

### Rescue of aging-associated decline in *Dnmt3a2* expression restores cognitive abilities

Oliveira, A.M. *et al.* *Nat. Neurosci.* doi:10.1038/nn.3151 (1 July).

The authors now provide a link between aging-related cognitive decline and hypomethylation by showing that aging in mice is associated with decreased expression of the DNA methyltransferase *Dnmt3a2* in the hippocampus. Restoration of *Dnmt3a* expression rescued the aging-associated cognitive function.

# Epigenetic mechanisms in neurological disease

Mira Jakovcevski & Schahram Akbarian

**The exploration of brain epigenomes, which consist of various types of DNA methylation and covalent histone modifications, is providing new and unprecedented insights into the mechanisms of neural development, neurological disease and aging. Traditionally, chromatin defects in the brain were considered static lesions of early development that occurred in the context of rare genetic syndromes, but it is now clear that mutations and maladaptations of the epigenetic machinery cover a much wider continuum that includes adult-onset neurodegenerative disease. Here, we describe how recent advances in neuroepigenetics have contributed to an improved mechanistic understanding of developmental and degenerative brain disorders, and we discuss how they could influence the development of future therapies for these conditions.**

In a living cell, the functional definition of the human genome goes far beyond its linear sequence of 6 (or when haploid, 3) billion base pairs. The epigenome ('epi-' is derived from the Greek for 'over' or 'above') has a rich cache of highly regulated structural modifications of DNA cytosine, histone residues and histone variants, and, as such, defines the moldings and three-dimensional structure of the genomic material in the cell nucleus and provides the molecular bridge between genes and the environment. The epigenome is also responsible for orchestrating the myriad transcriptional units, condensed chromatin clusters and other features that distinguish the various cell types and the states of development or disease that share a genome within an individual (Boxes 1 and 2; Fig. 1).

In this review, we describe how the field of epigenetics is reshaping the current thinking about neurological and neurodegenerative diseases. Only a few years ago, this field's primary focus was on a single mark—DNA methylation—in the context of cell division and early development. At first glance, these topics seemed to bear little relevance to the human postnatal or adult brain, which has a large proportion of postmitotic and highly differentiated cells and in which the majority of neurons develop, differentiate and permanently exit the cell cycle many weeks before birth. However, three recent major developments have prompted reconsideration of the potential importance of epigenetics in brain development and disease. First, studies in humans have indicated that the epigenetic landscape remains 'plastic' throughout all periods of brain development and aging and that ongoing dynamic regulation occurs even in neurons and other postmitotic constituents of the brain<sup>1–4</sup>. Second, disordered chromatin organization and function have been ascribed key pathogenic roles not only in several neurodevelopmental syn-

dromes of early childhood, but also in a subset of adult-onset hereditary neurodegenerative disorders<sup>5,6</sup>. Third, a rapidly expanding repertoire of chromatin-modifying drugs has shown unexpected therapeutic potential for a wide range of degenerative and functional disorders of the nervous system<sup>7–11</sup>. These three lines of discovery have now coalesced, igniting an enormous interest in the chromatin-associated mechanisms of neurological disease and providing the foundations for a new discipline: 'neuroepigenetics'<sup>12</sup>.

## Epigenetics and the mature brain

Although the focus of this review is on epigenetic changes in various neurological diseases, the first issue to consider is whether the normal course of maturation and aging is associated with changes in the brain's epigenome (Box 1). The hypothesis that aging is associated with epigenetic changes in the brain is attractive, given that there are widespread age-related changes in gene expression in the cerebral cortex, including the downregulation of many neuronal genes<sup>13,14</sup>. However, unlike the accumulation of somatic mutations and other structural DNA changes in the brain that affect promoter function during aging (and are likely to be irreversible)<sup>15</sup>, most or perhaps all epigenetic markings studied to date (Box 1) are now thought to be reversible, and there is no *a priori* reason for the unidirectional accumulation of a specific epigenetic mark in aging brain chromatin. Nonetheless, an increasing body of literature indicates that a substantial reorganization of the brain epigenome occurs during postnatal development and aging. The human cerebral cortex, for example, shows complex and gene-specific changes in the amounts of 5-methylcytosine (5-mC; cytosines are methylated at the 5 position carbon), and there is a steady rise in 5-mC at many promoters that continues into old age in conjunction with subtle changes (mostly declines) in the expression of transcripts originating from these promoters<sup>2,3</sup>. Such age-related epigenetic drift could affect an individual's vulnerability to neurodegenerative disease. For example, in mouse cerebellum, the levels of the 5-mC derivative hydroxymethylcytosine (5-hmC; Box 1), are subject to a tenfold increase between postnatal week 1 and adulthood<sup>16</sup>. Notably,

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among the genes that showed an increase in the amount of 5-hmC at their promoter during cerebellar maturation, pathways for aging-related neurodegenerative diseases and angiogenesis were overrepresented and included at least 15 genes linked to hereditary forms of spinocerebellar ataxia, a neurological syndrome defined by severe motor dysfunction with the degeneration of cerebellar Purkinje neurons and other systems<sup>16</sup>. It is also of interest that ten-eleven translocation (TET) proteins are responsible for converting 5-mC to 5-hmC; the active domains of these proteins belong to the same dioxygenase superfamily as hypoxia-inducible factor, an oxygen sensor that has been ascribed a key role in angiogenesis and oxidative-stress responses<sup>16</sup>. TET proteins may also function as oxygen sensors, thereby linking the epigenetic status of brain cells to (mal-)adaptive processes related to impaired tissue perfusion and oxygen supply.

In addition to changes in DNA methylation during development and aging, dynamic changes to the epigenetic landscapes of histone post-translational modifications (PTMs) can also occur. An age-dependent regulation of histone-methylation markings that can differentiate between open and repressive chromatin has been documented for the human prefrontal and cerebellar cortex<sup>17</sup>, and hundreds of loci undergo substantial chromatin remodeling in cortical neurons between infancy and advanced age<sup>4</sup>. Furthermore, the brains of SAMP8 mice, which are prone to accelerated senescence and have learning and memory deficits, show age-related drifts in histone PTMs. These epigenetic drifts are defined by a loss of the markings associated with active gene expression, such as monomethyl histone H4 lysine 20 (H4K20me1) and H3K36me3 (Box 1 and Fig. 1), in conjunction with a robust rise in the repressive mark H3K27me3 (ref. 18). Likewise, in the hippocampi of 16-month-old wild-type mice, genomic regions associated with actively expressed genes show a robust decline in acetylated H4K12 (ref. 19), a PTM that, like H3K36me3, is linked to the transcriptional elongation process<sup>20</sup>. In addition, upregulation of H4K12ac induced by histone deacetylase

inhibitors substantially improves hippocampal-dependent learning and memory in aged mice<sup>19</sup>. It is possible that age-related drifts in brain epigenomes negatively affect neuronal<sup>10,15</sup> and oligodendroglial<sup>21</sup> transcriptomes, thereby contributing to a decline in the signaling capacity of nerve cells, defects in axon myelination and other molecular alterations that have been linked to various cognitive disorders of the adult brain with and without neurodegeneration<sup>14,22</sup>. Together, these findings leave little doubt that brain epigenomes are indeed subject to dynamic changes throughout all periods of maturation and aging, and this may have important implications for the neurobiology of disease.

### Monogenic neurological diseases associated with epigenetic changes

Chromatin remodeling and the proper assignment of epigenetic marks on the genome are of fundamental importance for brain ontogenesis. These processes are also key control points in the stepwise transition from pluripotency to neural precursors to terminally differentiated neurons and glia<sup>23</sup>, and they are involved in developmental events such as neuronal migration and connectivity formation<sup>24</sup>. Perhaps unsurprisingly, more than a dozen neurological syndromes so far have been linked to mutations in single genes that encode DNA methyltransferase and histone modifying enzymes or their 'reader' proteins (Table 1). Notably, however, this list includes not only embryonic defects and multiorgan syndromes but also specific neurological disorders that show symptom onset in early childhood (for example, Rett syndrome) or neurodegeneration and regression beginning after adolescence (as in some cases of Kleeftstra syndrome) or as late as the third or fourth decade of life (for example, hereditary sensory and autonomic neuropathy type 1, (HSAN1) with early-onset dementia; Table 1). The molecular defects in these rare monogenic causes of intellectual disability and adult-onset dementia could be used as a starting point to unravel the underlying

## BOX 1 The organization and composition of the epigenome

The basic unit of chromatin is the nucleosome, which consists of 146 base pairs (bp) of genomic DNA wrapped around an octamer composed of the core histones H2A, H2B, H3 and H4. Chromatin fibers are defined as arrays of these nucleosomes, which are connected by linker DNA and linker histones (Fig. 1). The combined set of DNA and histone modifications and histone variants provide the major building blocks for the epigenome<sup>117,118</sup>.

**DNA methylation.** Two related, but functionally very different, types of DNA modification—methylation and hydroxymethylation of cytosine carbon 5, mostly in CpG dinucleotides—occur primarily within CpG-enriched islands (which are often defined by a GC percentage >50% across a minimum of 200 bp)<sup>119</sup>. The 5-mC and 5-hmC markings show a notably different distribution, with 5-hmC mostly confined to the 5' ends of genes in amounts that correlate overall with gene-expression activity<sup>120,121</sup>. In contrast, only a minute portion (<3%) of 5-mC locates to CpG islands at the 5' ends of genes, where it is thought to function as a repressive mark; the remaining 97% is found in intra- and intergenic sequences and within DNA repeats<sup>122</sup>.

**Post-translational histone modifications.** According to recent studies, the number of residue-specific amino acid PTMs in a typical vertebrate cell may be as high as 130 (ref. 123). PTMs include mono-, di- and trimethylation, acetylation and crotonylation, poly(ADP)-ribosylation and small-protein modification (by ubiquitin and small ubiquitin-like modifier, for example) of specific lysine residues, as well as arginine methylation and citrullination, serine phosphorylation, tyrosine hydroxylation and several others<sup>123–125</sup>. Various combinations and interrelations of these site- and residue-specific PTMs show close association with the functional architecture of chromatin, and specific epigenetic signatures have been identified for proximal promoters and gene bodies at sites of actual or potential transcription for enhancer and other regulatory sequences (Fig. 1) and for condensed and silenced chromatin<sup>126</sup>.

**Histone variants.** Apart from the core histones H2A, H2B, H3 and H4, metazoan genomes encode a number of histone variants that provide another layer of epigenetic regulation. Some well-known variants include H3.3, H2A.Z and H2A.X, which, in contrast to the canonical histones, are subject to replication-independent expression and assembly<sup>127</sup> and have strong effects on nucleosome stability and compaction<sup>128</sup>.

**Linker histones.** These histones, such as H1, are crucial for the three-dimensional architecture of chromatin and the 'zigzag' arrangement of nucleosomes as regulators of linker-DNA folding, and the amounts of linker DNA strongly correlate with nucleosome repeat length<sup>129</sup> (Fig. 1). Notably, the amounts of H1 in neurons are much lower than in most other cell types<sup>129</sup>, which explains the observation that the average nucleosome repeat length in rat neurons is 40 bp shorter than in glia (162 versus 201 bp)<sup>130</sup>. These neuron-specific features could have implications for disease vulnerability, because the RTT-associated protein MeCP2 competes with H1 for linker-DNA binding sites<sup>131</sup> and, furthermore, H1 levels are markedly increased in MeCP2-deficient brains<sup>36</sup>.

pathophysiology of these diseases, which could then open up hitherto unexplored therapeutic avenues for these specific conditions and eventually, perhaps, for a broader spectrum of neurological disease.

**Mutations affecting DNA methylation and neurological disease.** Hypomorphic (partial loss-of-function) mutations in *DNMT3B*, which encodes a DNA methyltransferase, are responsible for immunodeficiency, centromere instability and facial anomalies syndrome 1 (ICF1), a multiorgan syndrome that includes mental retardation and defective brain development<sup>25,26</sup>. Cultured cells from subjects diagnosed with ICF show imbalances in repressive (5-mC and H3K27me3) and facilitative markings (H3K4me3 and H3K9ac) at pericentric DNA repeats<sup>26</sup> (those at the 'middle' portions of the chromosome that surround the centromere) and at hundreds of promoters throughout the genome (Fig. 1). This imbalance is thought to contribute to the dysregulated expression of genes important for brain development, immune defense and many other key functions<sup>27</sup>. Hypomethylation defects also define ICF2, a nearly identical syndrome, but they occur at a different set of pericentric repeat sequences (Fig. 2). ICF2 is caused by mutations in *ZBTB24*, which encodes a putative transcriptional repressor<sup>28,29</sup>.

Mutations and structural variants in the X-linked gene *MECP2*, which encodes a methyl-CpG-binding protein, have been linked to Rett syndrome (RTT), a disorder of early childhood that has an incidence of 1 in 10,000 and is associated with developmental and cognitive regression as well as a broad range of neurological symptoms<sup>30</sup>. Males with RTT typically succumb during the perinatal period, but females survive, as they are somatic mosaics for the *MECP2* gene owing to X-chromosome inactivation. Individuals with RTT usually appear grossly normal at birth and then develop symptoms typically before 2 years of age. Since the discovery of *MECP2* mutations as a cause of RTT<sup>30</sup>, loss-of-function mutations and copy-number increases of the gene have been linked to various neurodevelopmental diseases, including childhood-onset schizophrenia and autism<sup>31</sup>. Studies in *Mecp2* mutant mice have provided insights into an unexpectedly complex molecular pathophysiology and linked specific neurological RTT phenotypes to transcriptional regulation at specific promoter sequences (Box 3, Fig. 3). However, as with ICF, the chromatin pathology in RTT goes far beyond dysregulated promoter activity, and it is likely to involve pericentromeric repeats and other heterochromatic domains. For example, many individuals with RTT show decreased clustering of the chromocenters (which represent clusters of highly condensed chromatin) inside the nucleus, and *Mecp2*-deficient neurons in mice do not show the expected chromocenter expansions after induction of neuronal activity<sup>32,33</sup>. Neuronal chromatin—which contains much higher amounts of MeCP2 than do glia<sup>34,35</sup>—shows a global disorganization in *Mecp2*-deficient brains, with supranormal amounts of the linker histone H1, hyperacetylation of nucleosome core histones, and derepression of transcriptional activity at pericentromeric repeats, retrotransposons and other normally silent repeats<sup>36</sup>. Alterations in the amount of

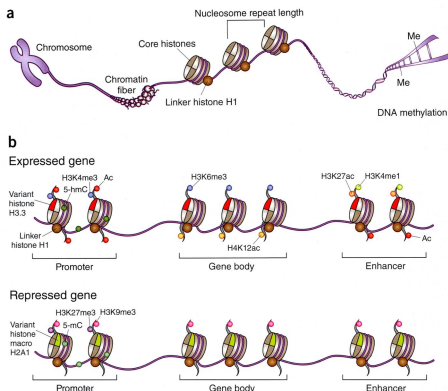


**Figure 1** The epigenome and chromatin organization. (a) Chromosomes are organized into domains of loose (euchromatin) or highly condensed (heterochromatin) chromatin and other loosely defined higher-order structures (such as 'globules'), some of which are tethered to the nuclear membrane. An 11-nanometer 'beads-on-a-string' chromatin fiber is comprised of nucleosomal arrays connected by linker DNA and linker histones, which function as major regulators of nucleosomal repeat length. Me, methyl group. (b) The distribution of DNA methylation and a small subset of ~130 post-translational histone markings, linker histones and core histone variants represents different regulation at active promoters, gene bodies and enhancers (top) as compared to silenced and repressed chromatin (bottom).

MeCP2, which could compete with H1 for occupancy of linker DNA<sup>36</sup>, could also affect nucleosome repeat length and the general architecture of chromatin fibers (Fig. 1 and Box 1).

It was recently discovered that monogenic brain disorders associated with defective DNA methylation are not limited to neurodevelopmental diseases; they also include some cases of HSAN1 (ref. 5), a rare neurodegenerative condition characterized by various neuropathies and onset of dementia in the third or fourth decade of life. The mutations associated with this condition are in the coding sequence of *DNMT1* (encoding DNA (cytosine-5)-methyltransferase-1)—specifically, within the targeting sequence domain that is important for the nuclear localization and preferential enrichment of Dnmt1 at pericentric and other repeat DNA sites and for Dnmt1 activity and stability<sup>5</sup>. Indeed, peripheral blood cells from individuals with HSAN1 showed hypomethylation at DNA repeat sequences and subtle methylation imbalances (mostly hypomethylation) at hundreds of gene promoters<sup>5</sup>. Furthermore, additional mutations in close proximity to the targeting sequence domain of *DNMT1* are found in some kindreds with autosomal dominant cerebellar ataxia, deafness and narcolepsy (ADCA-DN), which is also associated with early-onset dementia<sup>6</sup>. At first glance, it is surprising that these loss-of-function mutations in *DNMT1* selectively affect postmitotic neurons. Dnmt1 functions as part of the DNA replication machinery during the S phase of the cell cycle and tracks the replication fork to remethylate the newly synthesized DNA strands<sup>37</sup>, mechanisms considered relevant only for dividing cells. However, Dnmt1 may also play a part in maintaining



**Table 1** Monogenic brain disorders associated with DNA methylation and histone-modification defects

| Gene                                               | OMIM (gene)       | Function                                                                                            | Syndrome(s)                                                                                                                                                                                       | OMIM (phenotype)                  |
|----------------------------------------------------|-------------------|-----------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------|
| <i>ATRX</i> (Xq21.1)                               | 300032            | Replication-independent nucleosome remodeling and histone H3.3 incorporation                        | <i>ATRX</i> ; autism <sup>40</sup>                                                                                                                                                                | 301040                            |
| <i>CREBBP</i> (16p13.3);<br><i>EP300</i> (22q13.2) | 600140;<br>602700 | Transcriptional coactivator; histone acetyltransferase                                              | Rubinstein-Taybi syndrome (RSTS) 1 and 2 (ref. 105)                                                                                                                                               | 180849 (RSTS1);<br>613684 (RSTS2) |
| <i>DNMT1</i> (19p13.2)                             | 126375            | DNA methyltransferase; disease mutations are associated with hypomethylated repeats and promoters   | Hereditary sensory and autonomic neuropathy type 1 with adult-onset dementia <sup>5</sup> ; ADCA-DN <sup>5</sup>                                                                                  | 614116                            |
| <i>DNMT3B</i> (20q11.21)                           | 602900            | DNA methyltransferase; disease mutations are associated with hypomethylation of pericentric repeats | ICF1 mental-retardation syndrome <sup>28,106</sup>                                                                                                                                                | 242860                            |
| <i>ZBTB24</i> (6q21)                               | 614064            | Transcriptional repressor and regulator of DNA methylation at pericentric repeats                   | ICF2 mental-retardation syndrome <sup>28,29</sup>                                                                                                                                                 | 614069                            |
| <i>KDM5C</i> (Xp11.22)                             | 314690            | Histone H3K4 demethylase                                                                            | X-linked mental retardation <sup>107</sup> ; autism <sup>108</sup>                                                                                                                                | 300534                            |
| <i>EHMT1</i> (9q34.3)                              | 607001            | Histone H3K9 methyltransferase                                                                      | Kleefstra syndrome (mental retardation) <sup>109</sup> ; schizophrenia <sup>110</sup> ; nonspecific psychiatric phenotypes and neurodegenerative disease in postadolescence period <sup>111</sup> | 610253                            |
| <i>NSD1</i> (5q35.2–q35.3)                         | 606681            | Histones H3K36 and H4K20 methyltransferase                                                          | Sotos syndrome (mental retardation) <sup>112</sup>                                                                                                                                                | 117550                            |
| <i>PHF8</i> (Xp11.22)                              | 300560            | Histone H3K9 demethylase and transcriptional activator                                              | X-linked mental retardation without cleft lip and/or palate (Siderius-Hamel) <sup>113,114</sup>                                                                                                   | 300263                            |
| <i>RPS6KA3</i> (Xp22.12)                           | 300075            | Serine/threonine kinase (of both histones and nonhistone proteins)                                  | Coffin-Lowry X-linked mental-retardation syndrome <sup>115</sup>                                                                                                                                  | 303600                            |
| <i>MECP2</i> (Xq28)                                | 300005            | Methyl-CpG-binding protein                                                                          | RTT and other neurodevelopmental syndromes; autism <sup>116</sup>                                                                                                                                 | 312750                            |

the amount of DNA methylation in quiescent and even postmitotic cells, as it remains enriched at pericentric repeats and other condensed portions of the genome after S phase is complete<sup>38</sup>. Because this cell cycle-independent function of *Dnmt1* is dependent on its having an intact targeting sequence domain<sup>38</sup>, one could speculate that the deleterious *DNMT1* mutations in individuals with HSN1 result in faulty chromatin remodeling at repeat sequences of differentiated cells, including neurons. Notably, mutant mice that are mosaic for a prenatal deletion of *Dnmt1* in neural precursors lose virtually all of their brain cells with DNA hypomethylation within 1 month after birth<sup>39</sup>, and it will be interesting to find out whether similar phenotypes emerge in animals in which *Dnmt1* mutations are limited to the portions of the targeting sequence domain that are mutated in HSN1 pedigrees.

It is remarkable that each of these neurological conditions involving dysregulated DNA methylation—ICF, RTT and HSN1—is associated with widespread chromatin defects across the genome of differentiated cells in the central nervous system, including pericentric and other repeat-rich domains that have, until now, been barely explored in the neurosciences. Future developments in this area will be very interesting to follow, and they are likely to shed more light on the role of epigenetic regulation of these repeat DNAs in healthy and diseased brains.

**Heritable brain disorders with histone defects.** Loss-of-function mutations in *ATRX*, which encodes a multifunctional chromatin regulator, are responsible for various X-linked mental-retardation syndromes<sup>40</sup>. *ATRX* protein expression increases progressively during brain development, and in mice, *Atrx* ablation results in excessive apoptosis and defective migration of young neurons<sup>40,41</sup>. One *Atrx* mutant mouse line, which has a deletion in the *ATRX* zinc-finger motif, is a genetic model for humans who have a milder form of X-linked mental retardation<sup>42</sup>. This mouse model shows impaired learning behaviors as well as elongated,

structurally abnormal dendritic spines (a classical neuropathological abnormality found in various neurodevelopmental conditions)<sup>43</sup> and defective synaptic signaling in the frontal cortex<sup>42</sup>. However, the molecular mechanisms that link *ATRX* mutations to these neurological phenotypes remain unclear. One possible mechanism involves H3.3, a histone H3 variant that was first associated with active transcription but has subsequently been linked to ribosomal<sup>44</sup>, telomeric and pericentric repeats<sup>45–47</sup> (Fig. 2). *ATRX*, together with its binding partner and histone chaperone, the death domain-associated protein Daxx, is essential for replication-independent H3.3 incorporation into nucleosomes<sup>45–47</sup>. It has been suggested that *ATRX* preferentially targets G-rich tandem repeats, which tend to form four-stranded, aberrant DNA structures called G-quadruplexes, and refolds them back into double-stranded DNA with a regular nucleosomal organization<sup>44</sup>.

Whether any of these mechanisms are relevant in the context of neurological disease remains to be investigated. Notably, however, there are at least three monogenic disorders that show prominent epigenetic dysregulation of repeat DNA, including the ICF syndromes and *ATRX* (Fig. 2). Further work will be required to clarify whether the observed chromatin defects at the sites of repeat DNA are indeed a causal factor for the disordered neurodevelopment common to these conditions. Furthermore, numerous deleterious mutations in genes encoding histone-modifying enzymes have been identified as monogenic causes of neurodevelopmental or neurodegenerative disease (Table 1).

#### Disordered chromatin in neurodegenerative disease

Historically, studies of chromatin structure and function—and epigenetically driven concepts in general—have received little attention from researchers exploring the pathophysiology of slowly progressing neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases. Two independent lines of discovery have now

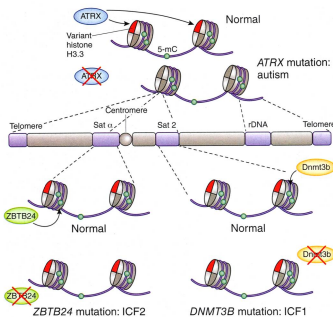


**Figure 2** Monogenic brain disorders with a heterochromatin defect.

Three examples of monogenic brain disorders associated with defects in heterochromatin are shown. Mutations in genes encoding Dnmt3b (associated with the disorder ICF1) or ZBTB24 (associated with the disorder ICF2) result in hypomethylation of different types of pericentric satellite (sat) repeats<sup>28,106</sup>. The multifunctional chromatin regulator ATRX controls the incorporation of the variant histone H3.3 not only into nucleosomes surrounding the transcription start sites of active genes, but also at various repeat sequences. Loss-of-function mutations in ATRX have been associated with X-linked mental-retardation syndromes.

DNA methylation at the promoters of several disease-associated genes, including the one encoding  $\alpha$ -synuclein itself<sup>49</sup>. There is additional, indirect, evidence that the delicate balance in the amounts of Dnmt1 and other DNA methyltransferases, including Dnmt3a, inside the nuclei of brain cells plays an important part in neuronal health and function. For example, the *in vivo* induction of apoptosis in motor neurons by toxic drugs or peripheral nerve lesions is associated with a preapoptotic rise in the amounts of Dnmt1 and Dnmt3a in the cell nucleus<sup>51</sup>. Furthermore, the amounts of methylcytosine and expression of DNMT are reportedly increased in pyramidal neurons in the cerebral cortex of subjects diagnosed with the sporadic motor-neuron disease amyotrophic lateral sclerosis<sup>51</sup>. Adding further complexity, there is evidence that Dnmt1 and Dnmt3a are not only targeted to the nucleus but also abundant in mitochondria, including those that are localized in distal neuronal processes and synapses<sup>51</sup>. Whether or not these DNA methyltransferases regulate mitochondrial functions in brain cells remains to be investigated, but preliminary evidence suggests that mitochondrial DNA, like nuclear DNA, is subject to methylation and hydroxymethylation of its cytosine residues<sup>52</sup>.

Likewise, histone-modifying enzymes could play a part in neurodegenerative disease. Because much of the evidence so far has been correlative, the neuroepigenetics field eagerly awaits further work to confirm (or refute) a key role for histone-modifying enzymes in the etiology or progress of neurodegenerative disorders. For example, the pathological sequestration of transcription factors vital for neuronal health, such as the cAMP response element-binding protein CREB and its binding partner CBP<sup>48,53</sup> (which encodes a histone acetyltransferase and is associated with Rubinstein-Taybi syndrome; **Table 1**), has also been linked to

moved epigenetics toward center stage in these fields. First, initial reports of the therapeutic benefits of histone deacetylase-inhibitor drugs in the nervous system used preclinical models for Huntington's disease, a hereditary condition caused by the excessive expansion of a CAG repeat in the huntingtin gene<sup>48,49</sup>. Second, the genetic findings described above link rare types of adult-onset dementia associated with hereditary neuropathy<sup>5</sup> or cerebellar ataxia<sup>6</sup> to deleterious mutations in proteins that regulate DNA or histone methylation. These findings may imply that other, more common types of adult-onset neurodegenerative disease could also be related to the defective regulation of chromatin in the brain.

Indeed, 'synucleinopathies' such as Parkinson's disease and dementia with Lewy bodies are associated with loss of Dnmt1 from the cell nuclei in the brain, both in patients with these conditions and in transgenic mice that overexpress synuclein<sup>50</sup>. This loss leads to deregulation of

## BOX 2 Readers, writers and erasers of chromatin marks

It is still under debate whether the epigenome's constituents play a causal part in establishing functional chromatin states. For example, the presence of open chromatin-associated histone modifications and variants at a specific locus in the genome is thought to reflect a process driven by transcriptional regulation and dynamic repositioning of nucleosomes. From this perspective, at least some DNA and histone modifications may function merely as 'cogs' in the chromatin-remodeling machinery rather than as key drivers<sup>132</sup>. Whatever the importance of DNA and histone modifications in regulating the chromatin-remodeling process, an increasing number of molecules that attach ('writers') or erase modifications to DNA or histone modifications or that bind ('readers') to a specific epigenetically modified site have emerged as key players in the pathophysiology and potential treatment of neurological disease. Most, or virtually all, epigenetic markings that have so far been studied in brain are subject to bidirectional and potentially highly dynamic regulation in the context of neuronal activity and various other paradigms<sup>133,134</sup>.

The underlying molecular machineries that contribute to the epigenome are often complex; for example, the DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b establish and maintain DNA-methylation marks. The actions of these enzymes are counterbalanced by active demethylation pathways involving 5-mC hydroxylation and oxidation via TET dioxygenases or by deamination of 5-mC or 5-hmC mediated by activation-induced deaminase and the enzyme APOBEC followed by base-excision repair-mediated replacement with (unmethylated) cytosine<sup>135,136</sup>. Other systems show a surprising degree of diversity, or perhaps redundancy, at the genetic level. For example, the various families of histone methyltransferases and demethylases together could easily account for >100 genes in a mammalian genome<sup>137,138</sup>. Proteins that bind to a specific epigenetic mark are defined by their characteristic reader module; well-studied examples include the methyl-CpG-binding domain for 5-mC-DNA, the bromodomain for lysine acetylation, the chromodomain and tudor, MBT, WD40-repeat and PHD-finger domains, which target methylated lysines or arginines in a residue-specific manner<sup>125</sup>. Conversely, specific methyl-lysine marks could be targeted by 50–100 reader proteins. For the open-chromatin mark H3K4me3, the reader proteins include many components of the RNA polymerase II-associated transcriptional initiation complex, whereas other marks, such as H3K9me3, are primarily targeted by transcriptional repressors and regulators of chromatin condensation<sup>139</sup>.

the  $\beta$ -amyloid plaques seen in the brains of individuals with Alzheimer's disease<sup>54</sup> and the polyglutamine aggregates and nuclear inclusions in Huntington's chorea<sup>55</sup>. Furthermore, a hyperactivity of tissue transglutaminase-2 in Huntington's disease leads to excessive nuclear amounts of lysine  $\epsilon$ -amino-glutamine  $\gamma$ -carboxamide bonds, which are resistant to proteolysis<sup>56</sup>. This excess results in an abnormal configuration of nuclear actin-cofilin complexes<sup>57</sup> and potentially abnormal histone polyamination, which leads to defective expression of PGC1- $\alpha$ , a transcription factor with an essential role in mitochondrial biogenesis<sup>58</sup>. Furthermore, excessive H3K9 methylation<sup>59</sup> and increased expression of macroH2A1, a variant histone broadly associated with repressive chromatin remodeling<sup>60</sup>, have been observed in blood and brain tissues from individuals with Huntington's disease, including the striatum and frontal cortex, which are heavily affected by the underlying neurodegenerative process in this disease. Such changes, in conjunction with additional defects in nucleosomal structure and function, may contribute to the decreased expression of the neurotrophic factor BDNF, dopamine receptors, MAP kinase signaling components and other transcriptional changes that have been observed in the striata of people with Huntington's disease<sup>61</sup>. Clearly, the hypothesis that histone-modifying enzymes are involved in the pathophysiology of neurodegenerative diseases warrants further investigation.

### Epigenetic targets in the treatment of neurological disease

**Histone acetylation.** There is great enthusiasm in academia and industry about the development of new epigenetic therapies, and this has been

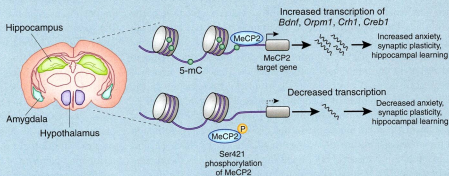
further boosted by the recent US Food and Drug Administration (FDA) approvals for the potent histone deacetylase inhibitors (HDAC inhibitors) suberoylanilide hydroxamic acid (SAHA, trade name Vorinostat) and romidepsin (trade name Istodax) in the treatment of hematological malignancies. Indeed, a large body of preclinical work has suggested that HDAC inhibitors could have therapeutic potential in a wide range of neurological conditions. These include acute brain injury and stroke paradigms, various neurodegenerative conditions such as Parkinson's and Alzheimer's disease, motor-neuron and triplet-repeat diseases such as Huntington's and spinocerebellar ataxias<sup>8–11</sup> and depression and other psychiatric illnesses<sup>62–64</sup> (Table 2). But it is still unclear whether HDAC inhibitors would benefit the patients affected by any of these conditions.

Moreover, like their counterparts—the histone acetyltransferases, or HATs—class I and II HDACs (commonly defined by their zinc-containing catalytic domain) and the class III HDACs or sirtuins (which are nicotinamide NAD<sup>+</sup> dependent) regulate lysine acetylation of non-histone proteins<sup>65,66</sup>. Indeed, nonhistone targets of sirtuin 1 (SIRT1) are thought to mediate its neuroprotective and cognitive phenotypes<sup>67,68</sup>. Such promiscuity for their target proteins adds further complexity to the interpretation of the therapeutic potential of HDACs that has emerged in models of neurological disease. Consider the example of frontotemporal dementia (FTD), a genetically heterogeneous neurodegenerative condition that primarily affects the frontal lobes and is associated with at least three different types of inclusion bodies in brain tissue<sup>69</sup>. Haploinsufficiency for *GRN*, which encodes progranulin, underlies a

### BOX 3 The complex pathophysiology of MeCP2

Among the growing list of genes associated with a brain-specific chromatin disorder, the largest share of the literature relates to the gene *MECP2*. The following discussion on emerging views of *MECP2*-associated transcriptional (dys-)regulation exemplifies the challenges and complexities in the endeavor to identify and pinpoint the molecular pathology of even a monogenetic disease.

Studies in both humans and mice have shown that phenotypic consequences arise when the amounts of functional MeCP2 protein drop below or rise above its normal amounts in brain<sup>140</sup>. Recent comparisons of the transcriptomes from gain- and loss-of-function *MeCP2* mutant mice to those from wild-type mice have provided some insights into



Fine-tuning of MeCP2 function and higher-order behavior. In adult mouse forebrain, promoter-bound MeCP2 upregulates the expression of key regulatory factors that control emotional and affective states, learning and memory. *In vivo*, phosphorylation (P) of MeCP2 Ser421 decreases promoter binding and gene expression, with corresponding changes in behavioral states. These observations were established by work in the amygdala (blue), hypothalamus (purple) and hippocampus (green) of *MeCP2* mutant and wild-type mice.

the complexities of MeCP2-mediated transcriptional regulation at specific promoter sequences. These studies have also implicated specific transcriptional changes in some of the emotional and behavioral changes that have been observed across the clinical spectrum of *MECP2* mutations. For example, in the basal forebrain, including the amygdala, more than 32 genes with established roles in the regulation of anxiety and social behaviors—including the neuropeptide corticotropin-releasing hormone (*Crh*) and the G protein-coupled  $\mu$ -opioid receptor (*Oprr1*)—were highly sensitive to *MeCP2* gene dosage<sup>141,142</sup>. In particular, elevated anxiety in mice that overexpress *MeCP2* is thought to be directly related to increased MeCP2 binding and upregulated transcription at the *Crh* and *Oprr1* genes<sup>141</sup>. Conversely, decreased anxiety in some *MeCP2* loss-of-function mutants has been linked to gene-expression changes, including decreased expression of *Crh1*<sup>142</sup>.

Taken together, these results seem counterintuitive to initial descriptions of MeCP2's role as a transcriptional repressor; adding to the complexity, neuronal activity induces MeCP2 phosphorylation at multiple serine sites, thereby altering the protein's affinity to its target gene promoters<sup>143</sup>. *In vivo*, ablation of a phosphoserine site downstream of MeCP2's transcriptional repression domain (S421) enhanced binding of MeCP2 to the *Bdnf* promoter, resulting in increased neurotrophin expression and improved hippocampal learning and plasticity<sup>144</sup> (see Figure). Thus, a highly complicated and multilayered process is involved in fine-tuning MeCP2 expression, which in turn controls many genes that have key regulatory roles in higher-order behaviors.

**Table 2 Chromatin-modifying drugs as potential therapies for neurological disease**

| Drug type and mechanism                     | Representative compound(s)                     | Effects on central nervous system in preclinical models                                                                                                                                                                                                                                                                                                          | FDA approval for non-neurological condition |
|---------------------------------------------|------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------|
| Sequesters Dnmt at DNA replication fork     | 5-azacytidine; zebularine (nucleoside analogs) | Disrupted hippocampal learning and reward behavior <sup>87–90</sup> in wild-type mice and rats; conferred stroke protection in mice <sup>93,94</sup>                                                                                                                                                                                                             | Yes                                         |
| Dnmt active site inhibitor                  | RG 108                                         | Prevented cell death in a mouse model of motor-neuron disease model <sup>51</sup> ; impaired hippocampal learning in rats <sup>52</sup>                                                                                                                                                                                                                          | No                                          |
| Class I/II HDAC inhibitor                   | Phenylbutyrate; SAHA; TSA                      | Had a broad neuroprotective profile in acute and chronic injury and neurodegeneration models, including ischemia, Huntington's and other polyglutamine and triplet diseases, and in a Parkinson's disease model (MPTP toxicity) <sup>8–11</sup> ; improved hippocampus-dependent learning and cognition and exerted antidepressant-like effects <sup>62–64</sup> | Yes                                         |
| Class III HDAC inhibitor                    | Nicotinamide; AGK2                             | Reduced amounts of phosphorylated tau and improved cognition in hippocampal learning tasks in a mouse model of Alzheimer's disease <sup>73</sup> ; reduced $\alpha$ -synuclein toxicity and dopaminergic cell death in a <i>Drosophila</i> model of Parkinson's disease <sup>74</sup>                                                                            | No                                          |
| Histone methyltransferase G9a/G9a inhibitor | BIX-01294                                      | Enhanced reward behavior in mice after exposure to a stimulant drug (cocaine) <sup>85</sup>                                                                                                                                                                                                                                                                      | No                                          |
| p300 histone-acetyl transferase inhibitor   | C646                                           | Reduced amounts of tau and neurotoxicity in cultured rat neurons and cells from people with Alzheimer's disease <sup>71</sup>                                                                                                                                                                                                                                    | No                                          |
| Topoisomerase I inhibitor                   | Topotecan; irinotecan                          | Reversed imprinting-mediated silencing of <i>Ube3a</i> (associated with Angelman's syndrome) in mouse brain <sup>96</sup>                                                                                                                                                                                                                                        | Yes                                         |
| Topoisomerase II inhibitor                  | Etoposide; dexrazoxane                         | Reversed imprinting-mediated silencing of <i>Ube3a</i> in mouse brain <sup>96</sup>                                                                                                                                                                                                                                                                              | Yes                                         |

type of FTD that is defined by brain inclusions containing TAR DNA-binding protein 43 (ref. 69). Notably, *GRN* expression is robustly upregulated by SAHA, which has been suggested as a promising treatment for FTD<sup>70</sup>. In contrast, FTD with parkinsonism-17 (FTDP-17) is defined by the abnormal accumulation, caused by excessive acetylation, of the microtubule-binding protein tau in the brain<sup>71</sup>. Thus, blocking of tau acetylation by inhibiting CBP and promotion of tau deacetylation via activation of SIRT1 has been proposed as a new therapeutic avenue to treat FTDP-17 and other 'tauopathies' such as Alzheimer's disease<sup>71</sup>. Upregulation of SIRT1 also suppresses  $\beta$ -amyloid production in the brains of mouse models of Alzheimer's disease<sup>72</sup>. Notably, however, the pan-sirtuin inhibitor nicotinamide also has therapeutic benefit in a transgenic Alzheimer's mouse model that overexpresses tau<sup>73</sup>. Although nicotinamide is a fairly broadly acting drug that has many effects beyond sirtuin inhibition, a subset of SIRT2-specific inhibitors elicits therapeutic benefits in cultured human cells and in *Drosophila* models of Parkinson's disease<sup>74</sup>. Thus, depending on the type of neurodegenerative disease and the specific HDACs and HATs involved, modulation of HDAC and HAT activity could have very different and potentially opposing clinical implications. Furthermore, although HDAC inhibitors are generally considered to promote neuronal growth and differentiation, there is also evidence that they could have potentially detrimental effects on the orderly maturation of astrocytes and oligodendrocytes<sup>75–77</sup>. Therefore, caution is warranted in evaluation of the potential benefits of these drugs in multiple sclerosis<sup>76</sup>, white-matter ischemia<sup>7</sup> and other neurological conditions that involve the damage or injury of the brain's non-neuronal cellular constituents. In addition, activation of retrotransposons that are normally epigenetically silenced could be another potentially detrimental side effect of HDAC inhibitors<sup>78</sup> (Box 4).

In addition to HAT and HDAC activators or inhibitors, other types of drugs that interfere with histone acetylation pathways have recently emerged. For example, the bromodomains of the BET protein family, which are classical 'readers' of acetylated chromatin and facilitate transcriptional activation<sup>79</sup>, can be targeted by highly specific small-molecule inhibitors that have been shown to correct transcriptional dysregulation in hematopoietic and solid malignancies<sup>80,81</sup>, inflammation and other medical conditions previously shown to be HDAC inhibitor sensitive<sup>82</sup>.

Because multiple BET reader proteins, including BRD1, BRD2 and BRD3, show robust and widespread expression in the mature mouse brain<sup>83</sup> (<http://www.brain-map.org/>), it will be interesting to explore bromodomain inhibitors in the context of some of the neurological disease models discussed above. As with the drugs that target HATs and HDACs, the specificity and side-effect profiles of bromodomain inhibitors will also require additional investigation.

**Histone methylation.** The clinical potential of drugs that can interfere with the regulation of histone methylation is also a promising, albeit largely unexplored, field. There are probably up to 100 histone methyltransferases and demethylases encoded in the human genome, and many of these enzymes are defined by functional domains outside the catalytic sites that are thought to contribute to target specificity and genomic occupancy patterns<sup>84</sup>. Like the HDAC inhibitors, a subset of histone methyltransferase inhibitors are in clinical trials for cancer treatment and are likely to be explored in the context of neurological disease in the near future<sup>85</sup>. An interesting candidate is the small molecule BIX-01294, which is an inhibitor for the histone H3K9-specific methyltransferases G9a and Glp<sup>86</sup>. This drug derepresses neuronal gene expression<sup>84</sup> and, when administered directly into the ventral striatum (a key structure in the brain's addiction circuitry), strongly enhances the development of reward behaviors in mice exposed to the stimulant drug cocaine<sup>85</sup>. It remains to be determined whether BIX-01294 elicits learning- and memory-enhancing effects outside of the stimulant addiction paradigm. However, it is interesting to note that the drug's mechanism of action could, at least in part, involve the inhibition of G9a- and Glp-mediated repressive chromatin remodeling at the promoters of *Bdnf*, *Cdk5*, *Arc* and other genes that function as key regulators for spine density and synaptic connectivity in the mouse brain<sup>85</sup>.

**DNA methylation.** Several inhibitors of DNA methylation, including the cytidine analogs 5-azacytidine and zebularine and nucleoside analogs that sequester Dnmt enzymes after being incorporated into DNA<sup>86</sup>, have been well characterized and are approved or are in preclinical and clinical trials for the treatment of cancer<sup>86</sup>. When administered directly into the brain tissue of mice and rats, these and other Dnmt inhibitors

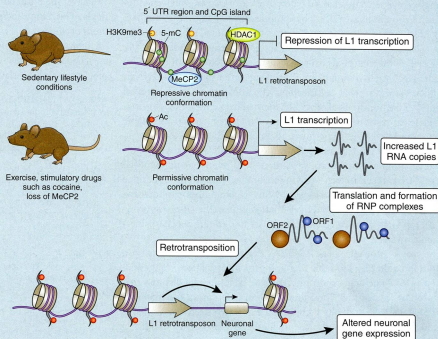


## BOX 4 Epigenetic control of the brain's 'jumping genes'

Retrotransposons, or 'jumping genes', are commonly defined as mobile elements that can proliferate in the germline of plants and mammals and thus contribute to the potential for massive genome expansion during the course of evolution<sup>145</sup>.

Approximately 40–50% of the human genome is derived from mobile elements and, more importantly, several classes of retrotransposons, which duplicate via RNA intermediates and then are inserted into a different site of the genome, remain active even in modern humans<sup>146</sup>. Some of the best-known examples of retrotransposons include LINE-1 (L1), Alu and SVA elements<sup>146</sup>, and it is probable that new insertions by these elements contribute to genome plasticity in individual brain cells<sup>147</sup>. In particular, the hippocampus and striatum are defined by much higher rates of *de novo* retrotransposition than other somatic tissues, such as blood, heart and liver<sup>147,148</sup>. It has been estimated that each hippocampal cell could harbor up to 800 new insertions, indicating a hitherto unexpected degree of genomic plasticity<sup>148</sup>. Notably, both L1- and Alu-based insertion events into human brain genomes disproportionately affect actively expressed genes, including many that are pivotal for synaptic transmission, such as those encoding the dopamine receptor DRD3, the amino acid transporters SLC6A5, SLC6A6 and SLC6A9 and the RAI1 transcription factor, which has been implicated in schizophrenia and 17p11.2 (Smith-Magenis) deletion syndrome<sup>147</sup>. Therefore, retrotransposon activity could contribute to neuronal diversity<sup>149</sup> and even drug- or experience-dependent plasticity<sup>150,151</sup> (Fig. 4).

Insertional mutagenesis could also have potentially detrimental consequences, such as the aberrant activation or repression of neighboring genes, exon shuffling and gene deletions<sup>149</sup>. Epigenetic regulators are vital for a cell's ability to suppress retrotransposon activity, and L1, Alu and other elements that utilize the L1 replication machinery are epigenetically silenced via repressive DNA methylation and histone modifications<sup>152</sup>. Therefore, when repressive chromatin remodeling at sites of mobile elements is 'exchanged' with transcriptional-activation complexes, as when the WNT3a- $\beta$ -catenin complex binds to the L1 promoter<sup>153,154</sup>, excessive amounts of insertional mutagenesis could cause cell damage. For example, loss of the repressive DNA-methylation reader protein MeCP2 in neural cultures or in the brains of mice with *MECP2* mutation results in a modest excess of L1 activity<sup>36,155</sup> in conjunction with changes in global chromatin states (see Figure). Increased L1 retrotransposition was also recently detected in cells from people with ataxia telangiectasia, an autosomal recessive neurodegenerative condition caused by alterations of the DNA damage sensor and the serine/threonine kinase ATM<sup>78</sup>. Loss of ATM results in increased reverse transcriptase efficiency, thereby fostering new L1 insertions in the genome<sup>78</sup>. It is conceivable that RTT and ataxia telangiectasia are merely the 'tip of the iceberg' and that numerous other neurological conditions with links to transposon dysregulation may emerge in the near future.



Epigenetic regulation of retrotransposon activity in the mouse nervous system. Epigenetic 'brakes' preventing excess L1 transcription in the brain include DNA methylation and repressive chromatin remodeling, which involve the actions of the histone deacetylase HDAC1 and the methyl-CpG-binding protein MeCP2. Physiological activities are associated with mild relaxation of L1 repression, and stimulant drugs of abuse or loss of MeCP2 could substantially upregulate the number of L1 RNA copies. The L1 RNAs are then translated and assembled into complexes for active retrotranspositions, thus resulting in genome toxicity through excess amounts of *de novo* genome integrations and changes in neuronal gene expression. RNP, ribonucleoprotein; ORF, open reading frame.

Merina Corral

disrupt synaptic plasticity and hippocampal learning and memory, and they are powerful modulators of reward and addiction behaviors<sup>87–90</sup>. But nucleoside analogs are thought to act primarily at sites of DNA synthesis and replication during the cell cycle; thus, they seem at first glance to be of little relevance to postmitotic neurons and glia. Notably, N-phenyl-L-tryptophan (RG 108) and other drugs that interfere with sites at which Dnm1 is active independently of DNA replication still elicit a robust impairment of hippocampal learning and memory<sup>91,92</sup>. The potential of drugs that interfere with Dnm1 activity may go beyond these examples of synaptic and behavioral plasticity. In mice, for example,

treatment with Dnm1 inhibitors can confer stroke protection after mild ischemia; furthermore, haploinsufficiency for *Dnm1* in mice is associated with smaller infarction volumes after acute ischemia and stroke<sup>93,94</sup>. A recent study reported a strong, antiapoptotic effect for RG 108 in an injury-based mouse model of amyotrophic lateral sclerosis<sup>51</sup>.

**Topoisomerase inhibitors.** Topoisomerases are DNA-cleaving enzymes that are important in the processes of replication and recombination, transcription and chromatin remodeling<sup>95</sup>. A recent study using an unbiased high-content screening approach in mouse primary cortical

neurons discovered that a diverse group of molecules that function as inhibitors of topoisomerase I or II can unlock the expression of the normally epigenetically silenced paternal allele of *Ube3a*, which encodes ubiquitin protein ligase E3A. These topoisomerase inhibitors mediate their effects by reducing the expression of the imprinted *Ube3a* antisense RNA (*Ube3a-ATS*)<sup>96</sup>. The expression of this antisense RNA is normally repressed on the maternal chromosome in conjunction with the allele-specific DNA methylation of an imprinting center (which is a DNA or chromatin structure that carries epigenetic information about parental origin)<sup>97</sup>. Like hundreds of other loci defined by parent-of-origin-specific gene expression, *Ube3a* has been considered epigenetically stable throughout life<sup>98</sup>. This hypothesis now needs to be revised, however, given that even a single intrathecal infusion of the FDA-approved topoisomerase inhibitor topotecan was sufficient to relieve silencing of the paternal *Ube3a* (sense) transcript in the lumbar spinal neurons of mice for at least 3 months<sup>99</sup>. The most obvious explanation for topotecan's mechanism of action—altered DNA methylation of the *Ube3a* imprinting center—has been ruled out. Thus, the underlying mechanism(s) remain a mystery.

The reactivation of paternal *UBE3A* expression via topoisomerase inhibition could provide a starting point for investigation of potential therapies for Angelman syndrome, a neurodevelopmental disorder that is caused by loss-of-function mutations and deletions at the maternal *UBE3A* locus<sup>99–101</sup> and for which there are now no effective treatments. Although it is not known at present whether topoisomerase-mediated reversal of imprinting-related gene expression is specific to the *UBE3A* locus, this issue could be addressed by, for example, fusing topoisomerase enzymes to customized motifs for sequence-specific binding at *UBE3A*. For example, chimeric zinc-finger nucleases or transcription activator-like (TAL) effectors of plant pathogenic bacteria have been fused to the FokI restriction enzyme, allowing the induction of 'custom-made' DNA strand breaks at specific and even at unique loci in the genome<sup>102,103</sup>. It is certainly worth exploring whether similar strategies could be used to create a chimeric protein comprised of a topoisomerase enzyme fused to a custom-made zinc-finger or TAL effector sequence that can specifically target the *UBE3A* imprinting center.

## Outlook

The field of neuroepigenetics is evolving at a rapid pace. We are beginning to understand more about the neural mechanisms that mediate the reversible and bidirectional regulation of DNA methylation and various histone modifications. Furthermore, as discussed in this review, the epigenetic dysregulation of gene expression and chromatin architecture could play a prominent part in the pathophysiology of various neurological disorders. However, in many of these disease conditions the mutant epigenetic regulator seemingly operates in a highly complex and multifunctional manner at a large number of genomic loci. For example, RIT1, ICF1, ICF2 and HSN1 are examples of a heterogeneous group of neurodevelopmental and neurodegenerative disorders caused by alterations in proteins that 'read' or 'write' DNA methylation, and each disease shows disordered DNA methylation at numerous single-copy genes as well as in specific sequences of repeat DNA in subdomains of constitutive heterochromatin. It will be important to clarify, for each disorder, which of the genomic loci affected are important for the manifestation, progression and potential treatment of disease, and which are merely bystanders, irrelevant to the disease process.

For the numerous chromatin-modifying drugs that have shown promise in preclinical studies, it will be important to pinpoint their key mechanisms of action. For example, are the beneficial effects of HDAC inhibitors in various acute and chronic neurodegenerative and cognitive disorders due to their broad effects on histone modifications, changes in

the acetylation of nonhistone proteins (such as Bruchpilot, a presynaptic molecule and regulator of vesicle release<sup>104</sup>) or a combination thereof? Whatever the underlying mechanism of action, we predict that, as in the fields of oncology and general medicine (which are now pursuing hundreds of clinical trials with epigenetic drug targets), the treatment options for neurodevelopmental and neurodegenerative diseases will soon be enriched by the array of chromatin compounds that are emerging from preclinical and translational research (Table 2).

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## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# Asic3 is a neuronal mechanosensor for pressure-induced vasodilation that protects against pressure ulcers

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**Pressure-induced vasodilation (PIV) delays the decrease in cutaneous blood flow produced by local application of low pressure to the skin, a physiologically appropriate adjustment of local vasomotor function. Individuals without a normal PIV response have a high risk of ulceration. Here we demonstrate that acid-sensing ion channel 3 (Asic3) is an essential neuronal sensor for the vasodilation response to direct pressure in both humans and rodents and for protecting against pressure ulcers in mice.**

Pressure ulcers are common in a variety of patient settings and are associated with adverse health outcomes and high treatment costs<sup>1-3</sup>. A local application of low pressure on healthy skin induces an increase

in cutaneous blood flow<sup>4</sup> that is altered by pathological conditions such as diabetes and aging<sup>5,6</sup>, which are known to increase the risk of pressure ulcer formation. Individuals lacking a normal PIV response show an early decrease of cutaneous blood flow to the application of very low pressures<sup>5,6</sup>, reflecting a vascular fragility of the skin that increases the risk of ulceration.

The cutaneous neuronal mechanosensor initiating the vasodilation against low local pressure<sup>4</sup> remains unknown. Asic channels<sup>7,8</sup> are good candidates to participate in mechanical transduction in PIV, although their role as neuronal mechanosensors is still being discussed<sup>9,10</sup>. Among the three neuronal Asic channels, Asic1, Asic2 and Asic3 (ref. 11), Asic3 is a particularly noteworthy candidate because (i) it is largely expressed in sensory neurons<sup>7</sup>; (ii) it resides in nerve endings at sites where mechanical stimuli are converted into electrical signals<sup>12</sup> and mechanosensitivity is altered to some extent in *Asic3* (officially known as *Acan3*)-knockout mice<sup>12,13</sup>; and (iii) it is present in a class of sensory neurons that co-express calcitonin gene-related peptide (CGRP), an important vasodilator peptide required for PIV<sup>4</sup>, and some of these sensory neurons innervate blood vessels<sup>14</sup>.

To test the possibility that Asic3 mediates PIV, we evaluated PIV properties in *Asic3*<sup>-/-</sup> and *Asic3*<sup>+/+</sup> mice. Local pressure application induced a PIV of  $41 \pm 3\%$  in *Asic3*<sup>+/+</sup> mice, whereas PIV was never

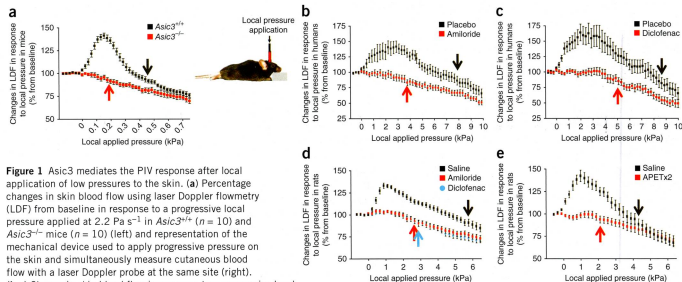


**Figure 1** Asic3 mediates the PIV response after local application of low pressures to the skin. (a) Percentage changes in skin blood flow using laser Doppler flowmetry (LDF) from baseline in response to a progressive local pressure applied at  $2.2 \text{ Pa s}^{-1}$  in *Asic3*<sup>+/+</sup> ( $n = 10$ ) and *Asic3*<sup>-/-</sup> mice ( $n = 10$ ) (left) and representation of the mechanical device used to apply progressive pressure on the skin and simultaneously measure cutaneous blood flow with a laser Doppler probe at the same site (right). (b,c) Change in skin blood flow in response to a progressive local pressure applied at  $11 \text{ Pa s}^{-1}$  in healthy individuals following intradermal injections of placebo ( $n = 20$ ) or amiloride ( $n = 20$ ) (b) or topical application of placebo ( $n = 20$ ) or diclofenac ( $n = 20$ ) (c). (d,e) Change in skin blood flow in response to a progressive local pressure applied at  $11 \text{ Pa s}^{-1}$  in rats following subcutaneous injections of saline ( $n = 10$ ), amiloride ( $n = 10$ ) (d) or subcutaneous injections of saline ( $n = 10$ ) or APETx2 ( $n = 10$ ) (e). Data are means  $\pm$  s.e.m. Arrows indicate the local pressure for which the skin blood flow significantly decreased from baseline (before pressure application) using one-way analysis of variance (ANOVA) and Dunnett's multiple comparison *post hoc* test.

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seen in *Asic3*<sup>-/-</sup> mice, leading to an early decrease of the cutaneous blood flow following the application of very low pressures (Fig. 1a).

The succession of steps leading to PIV<sup>4</sup> involves (i) pressure stimulation of specialized sensory neurons, (ii) release of CGRP from afferent sensory nerve endings in response to local pressure application, (iii) nitric oxide (NO) liberation from the endothelium in response to CGRP and (iv) vascular smooth muscle relaxation in response to endothelial NO. We found that normal vasodilator function of the endothelium and smooth muscle in *Asic3*<sup>-/-</sup> mice was intact on the basis of studies using acetylcholine and an NO donor, sodium nitroprusside (SNP), respectively (Supplementary Fig. 1a,b). These results indicate that the loss of PIV is due specifically to a failure of pressure detection.

A schematic diagram outlining the cascade of events by which we propose that activation of *Asic3* elicits PIV is presented in Supplementary Figure 2. In this model, *Asic3* activation triggers the release of the vasodilator peptide CGRP from afferent nerve terminals. Indeed, treatment of *Asic3*<sup>+/-</sup> mice with the CGRP antagonist  $\alpha$ CGRP(8–37) caused a similar defect in the PIV response as seen in *Asic3*<sup>-/-</sup> mice (Supplementary Fig. 3a).

We next used *Asic3* inhibitors to further test its role as a cutaneous mechanoreceptor in PIV in humans and rats. We used three different inhibitors of *Asic3* function: amiloride<sup>7,11</sup>, diclofenac<sup>11</sup> and APETx2 (ref. 11). Amiloride and diclofenac are widely used drugs in the clinic, but neither of them is specific for *Asic3*, whereas APETx2, a sea anemone peptide, is considered to be an *Asic3*-specific inhibitor<sup>11</sup>. In humans and in rats, PIV was abolished by either intradermal or subcutaneous injection of amiloride and by either topical application or subcutaneous injection of diclofenac (Fig. 1b–d and Supplementary Fig. 4), without changes in endothelium-dependent and endothelium-independent vasodilator responses (Supplementary Fig. 5a–d). In rats, PIV was abolished by local injection of APETx2 (Fig. 1e), indicating again that cutaneous *Asic3* is crucially involved in PIV. APETx2 had no effect on endothelium-dependent (assayed with acetylcholine) or endothelium-independent (assayed with SNP) vasodilator responses that could explain its abolition of PIV (Supplementary Fig. 5e,f). These pharmacological findings clearly confirm that *Asic3* inhibition leads to loss of PIV due to pressure detection failure.

We found that ischemic lesions in mice can be induced after application of higher pressures for longer times than used in the protocol to elicit PIV. Using a protocol in which a compression was applied for 4 h and ischemic lesions were evaluated 24 h later, the incidence and extent of these lesions were more pronounced in *Asic3*<sup>-/-</sup> mice as compared with *Asic3*<sup>+/-</sup> mice (Fig. 2a and Supplementary Figs. 6 and 7). Whereas 60% of *Asic3*<sup>+/-</sup> mice developed ischemic lesions, all of the *Asic3*<sup>-/-</sup> mice did. Moreover, the cutaneous area with a loss of perfusion was smaller in *Asic3*<sup>+/-</sup> mice ( $33 \pm 14 \text{ mm}^2$ ) than in *Asic3*<sup>-/-</sup> mice ( $144 \pm 26 \text{ mm}^2$ ,  $P < 0.01$ ), corresponding to  $19 \pm 8\%$  and  $81 \pm 15\%$  ( $P < 0.01$ ) of the total compressed area, respectively (Fig. 2a). Compression led to a severe ischemia in all mice but this persisted after compression release only in *Asic3*<sup>-/-</sup> mice, whereas cutaneous perfusion and oxygenation rapidly returned to basal values in *Asic3*<sup>+/-</sup> mice (Fig. 2b,c). The drastic decrease in reactive hyperemia in *Asic3*<sup>-/-</sup> mice was not due to defective function of the endothelium (assayed with acetylcholine) or smooth muscle (assayed with SNP) (Supplementary Fig. 1c,d), nor was it due to intravascular thrombosis, excluding an obstructive etiology for these ischemic lesions (Supplementary Figs. 6 and 7). As observed for the PIV response, CGRP blockade mimicked the effects of *Asic3* deletion on reactive hyperemia and to a lesser extent on ischemic lesions (Supplementary

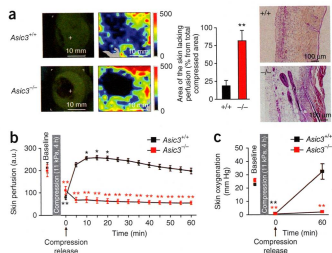


**Figure 2** The ischemic response to compression is exacerbated by *Asic3* deficiency. (a) Compression was applied for 4 h using a magnet (11 kPa), and the skin was evaluated 24 h after compression release. Typical examples of the general status of the skin using H&E staining and assessment of perfusion by laser Doppler imaging (in arbitrary units) in *Asic3*<sup>+/-</sup> and *Asic3*<sup>-/-</sup> mice (left); ischemic lesion size (the area lacking perfusion expressed as a percentage of the total compressed area ( $177 \text{ mm}^2$ ) in *Asic3*<sup>+/-</sup> mice ( $n = 5$ ) and *Asic3*<sup>-/-</sup> mice ( $n = 5$ ) (middle); and histology (right; H&E staining). (b) Skin perfusion (laser Doppler imaging) before application of the compressive ischemia (baseline) and over a time course of 60 min after compression release in *Asic3*<sup>+/-</sup> ( $n = 5$ ) and *Asic3*<sup>-/-</sup> mice ( $n = 5$ ). a.u., arbitrary units. (c) Skin oxygenation (transcutaneous pressure of oxygen at  $44^\circ\text{C}$ ) before compressive ischemia (baseline) and immediately after compression release (magnet removal) and 60 min later in *Asic3*<sup>+/-</sup> ( $n = 5$ ) and *Asic3*<sup>-/-</sup> mice ( $n = 5$ ). Data are means  $\pm$  s.e.m.  $^{**}P < 0.01$  versus *Asic3*<sup>+/-</sup> mice using Mann-Whitney *U* test (a);  $^{*}P < 0.05$ ,  $^{***}P < 0.01$  versus respective baseline values using one-way ANOVA and Dunnett's multiple comparison *post hoc* test (b,c).

Fig. 3b,c). Taken together, our results indicate that *Asic3*-containing channels are essential for PIV in both rodents and humans as well as for protection against ulcer formation in mice when a higher-pressure and longer-lasting compression was applied.

*Asic3* channels in the skin have two types of sensory roles. First, as a mechanosensor that triggers the appropriate microvascular responses through CGRP and produces PIV at low pressures; and second, as an acid sensor, as previously observed in both rodents<sup>7,11,12,15</sup> and humans<sup>16</sup>. When pressure applied to the skin increases in strength and in duration, it leads to ischemia associated with production of protons and lactate that can activate *Asic3* channels<sup>17</sup>. This acid- and lactate-induced *Asic3* activation seems to be essential for reactive hyperemia after compression. Thus, we propose that *Asic3* channel function has beneficial effects on the microcirculation and protects from pressure ulcers that represent a major medical problem<sup>2,3,18</sup>. The discovery of the essential role of *Asic3* for protection against pressure-induced lesions may offer new therapeutic directions.

## METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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#### AUTHOR CONTRIBUTIONS

B.F., J.L.S. and M.L. conceived of and designed the experiments, analyzed the data, and contributed to writing and editing the manuscript; B.F. performed the experiments; E.L. and D.S.-R. contributed to discussions and provided tools important for completion of the work.

#### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# Pathological neoangiogenesis depends on oxidative stress regulation by ATM

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The ataxia telangiectasia mutated (ATM) kinase, a master regulator of the DNA damage response (DDR), acts as a barrier to cellular senescence and tumorigenesis. Aside from DDR signaling, ATM also functions in oxidative defense. Here we show that Atm in mice is activated specifically in immature vessels in response to the accumulation of reactive oxygen species (ROS). Global or endothelial-specific *Atm* deficiency in mice blocked pathological neoangiogenesis in the retina. This block resulted from increased amounts of ROS and excessive activation of the mitogen activated kinase p38 $\alpha$  rather than from defects in the canonical DDR pathway. *Atm* deficiency also lowered tumor angiogenesis and enhanced the antiangiogenic action of vascular endothelial growth factor (Vegf) blockade. These data suggest that pathological neoangiogenesis requires ATM-mediated oxidative defense and that agents that promote excessive ROS generation may have beneficial effects in the treatment of neovascular disease.

Pathological angiogenesis is a hallmark of neovascular diseases, including ocular neovascularization and cancer. A growing number of antiangiogenic molecules have been discovered that can limit pathological angiogenesis, including VEGF blockers. Several of these are approved for clinical use and have substantial therapeutic efficacy<sup>1,2</sup>. However, VEGF blockade not only suppresses pathological angiogenesis but also damages healthy vessels, resulting in toxic side effects<sup>3,4</sup>. Moreover, a fraction of patients are refractory or acquire resistance to VEGF inhibitors, possibly through compensation by other proangiogenic mechanisms<sup>5,6</sup>. Therefore, there is a need to identify new proangiogenic signaling cascades that are specifically active in pathological angiogenesis. Inhibitors of these pathways could be combined with VEGF blockers to improve current antiangiogenic therapy<sup>7,8</sup>.

The DDR pathway is crucial in maintaining genomic stability and cellular homeostasis and acts as a barrier to cellular senescence and tumorigenesis<sup>9,10</sup>. It was recently reported that the loss of the H2A histone family member X (H2AX), a key protein in the DDR pathway, reduces ocular and tumor neovascularization<sup>11</sup>, suggesting the DDR pathway as a target for antiangiogenic therapy. The ATM kinase is another key player in the DDR pathway<sup>10,12</sup>. Mutations in the *ATM* gene cause the human hereditary disease ataxia telangiectasia, which is characterized by several typical symptoms, including hypersensitivity to radiation, predisposition to hematological malignancies, cerebellar ataxia, telangiectasia and immunodeficiency<sup>13</sup>. A role for ATM in vascular stability has long been suspected because of the manifestation of telangiectasia (dilated blood vessels) and vascular leakiness in both patients with ataxia telangiectasia and aged *Atm*-deficient mice<sup>12–14</sup>.

In this study we identify a role for ATM as a proangiogenic protein. However, this activity was caused not by effects on the DDR pathway, but rather through a separate effect on the oxidative activation of p38 $\alpha$ .

## RESULTS

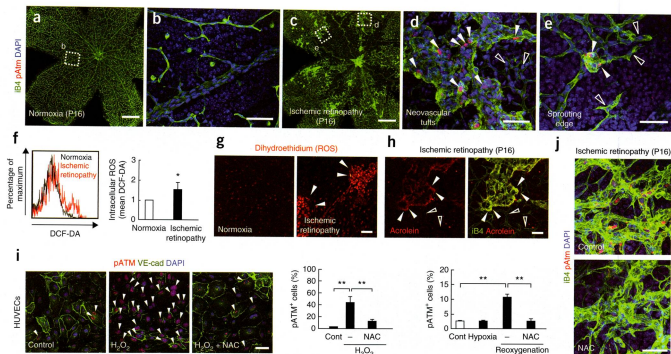
### ATM is activated in immature vessels in response to ROS

As a first step in studying ATM function in normal and pathological vessels, we examined *Atm* activation in mouse retinal blood vessels using an antibody that recognizes *Atm* phosphorylated at Ser1987 (pAtm) (Ser1981 is the human equivalent)<sup>15</sup>. In normally developing mice, we did not find pAtm expression in retinal vessels at postnatal day 16 (P16) (Fig. 1a,b and Supplementary Fig. 1). We then studied *Atm* activation in an ischemic retinopathy mouse model<sup>16,17</sup>. In this model, neovascular tufts, similar to tumor vessels, are exposed to stressed conditions. Excessive angiogenic factors are secreted by surrounding hypoxic cells<sup>17,18</sup> and the vessels are poorly perfused<sup>19</sup> and unstably undergo repeated cycles of hypoxia and reoxygenation. We found an abundance of pAtm in endothelial cells within pathologically expanding neovascular tufts in the ischemic retinopathy model but did not find pAtm in morphologically normal vessels surrounding neovascular tufts (Fig. 1c,d and Supplementary Fig. 1) and, in particular, did not find pAtm in endothelial tip cells migrating into the central avascular area (Fig. 1e and Supplementary Fig. 1). We detected only a negligible amount of reactivity to the pAtm antibody in the retinal vessels of *Atm*<sup>-/-</sup> mice (Supplementary Fig. 2a,c–f), showing specificity of the pAtm staining. We then measured oxidative stress levels using the dye 2',7'-dichlorofluorescein diacetate (DCF-DA), which detects cytosolic ROS. A subset of retinal endothelial cells isolated from wild-type mice with ischemic retinopathy, but not from control mice, was highly positive for DCF-DA fluorescence (Fig. 1f). Dihydroethidium staining on retinal whole mounts showed abundant ROS accumulation in neovascular tufts (Fig. 1g). We confirmed this finding by immunostaining with acrolein (Fig. 1h), a commonly used marker of lipid peroxidation resulting from oxidative stress<sup>20</sup>.

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To examine whether elevated ROS concentrations activate Atm in endothelial cells, we performed an *in vitro* assay. *In vitro* exposure to hydrogen peroxide markedly activated ATM in human umbilical vein endothelial cells (HUVECs) (Fig. 1i). Suppression of ROS accumulation by treatment with the antioxidant N-acetyl-L-cysteine (NAC) abrogated the hydrogen peroxide-induced activation of ATM in these cells (Fig. 1i). Although culturing the cells in 1% oxygen for 24 h did not affect ATM activation, this activation was enhanced when hypoxia was followed with 24 h of culture in 20% oxygen (reoxygenation) (Fig. 1i). The effect of reoxygenation was abrogated by supplementation with NAC (Fig. 1i), implicating ROS. Moreover, *in vivo* suppression of ROS accumulation in mice subjected to the ischemic retinopathy model by systemic treatment of with NAC abrogated ATM activation in neovascular tufts (Fig. 1j).

### Endothelial ATM is required for pathological angiogenesis

To explore the function of Atm in pathological angiogenesis, we induced ischemic retinopathy in Atm-deficient (*Atm*<sup>−/−</sup>) mice. These mice showed almost no formation of neovascular tufts, whereas revascularization into the vaso-obiterated area was enhanced compared to wild-type mice in this model (Fig. 2a–c). This effect of Atm deficiency stands in stark contrast to the effects of blocking either Vegf or Vegf receptor 2 (Vegfr2) in this model, which suppresses both the formation of neovascular tufts and the recovery of normal vessels<sup>21,22</sup>. Generally, the number of neovascular tufts correlates inversely with

revascularization into the central avascular area<sup>19</sup>. Therefore, we considered the possibility that loss of Atm primarily has effects through promoting normal vessel recovery rather than by inhibiting pathological vessel formation. The number of neovascular tufts was lower in *Atm*<sup>−/−</sup> mice compared to *Atm*<sup>+/+</sup> mice as early as P12, whereas the difference in avascular area between the two groups became significant at P16 (Supplementary Fig. 3a,b). This time lag suggests that neovascular formation is a primary target of Atm deficiency. The enhanced recovery of normal vessels in *Atm*<sup>−/−</sup> compared to *Atm*<sup>+/+</sup> mice at P16 might be the result of a higher expression of *Vegfa* at P14 in *Atm*<sup>−/−</sup> compared to *Atm*<sup>+/+</sup> mice (Supplementary Fig. 3c), which was presumably caused by increased tissue hypoxia resulting from fewer total vessels (Supplementary Fig. 3a). In addition, vascular leakage in *Atm*<sup>−/−</sup> retinas at P16 was milder than that in *Atm*<sup>+/+</sup> retinas (Fig. 2b), reflecting a lower number of neovascular tufts, which are composed of immature and leaky vessels.

In *Atm*<sup>−/−</sup> mice, endothelial cell proliferation was lower but apoptosis was higher than in *Atm*<sup>+/+</sup> mice (Fig. 2d). Endothelial cells isolated from *Atm*<sup>−/−</sup> mice (cells with vascular endothelial cadherin (VE-cadherin) expression after fluorescence-activated cell sorting (FACS)) showed less proliferation and more apoptosis than did the same population of cells from *Atm*<sup>+/+</sup> mice (Supplementary Fig. 4). Before hyperoxia challenge at P8, vascular structures appeared similar in *Atm*<sup>+/+</sup> and *Atm*<sup>−/−</sup> mice; moreover, immediately after hyperoxia (P11), the amount of vaso-obiterated area was also similar in these two strains (Fig. 2e).

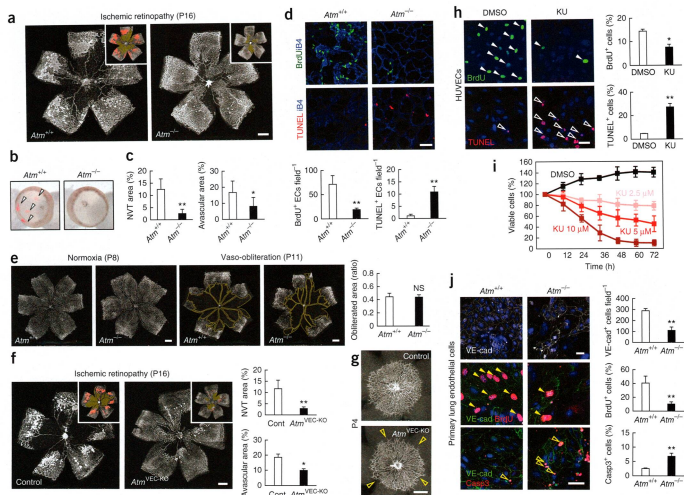


**Figure 2** Endothelial *Atm* is required for retinal pathological angiogenesis. **(a–c)** Isolectin staining **(a)** and stereomicroscopy **(b)** of whole-mount retinas from *Atm*<sup>+/+</sup> and *Atm*<sup>-/-</sup> mice with ischemic retinopathy at P16 and quantification of the neovascular tuft (NVT) and avascular areas ( $n = 8$  mice per genotype). **(c)** In the insets of **a**, the red area indicates NVTs and the yellow area indicates the avascular area. Open arrowheads in **b** indicate retinal hemorrhage. **(d)** Short-term BrdU incorporation assay and TUNEL staining in the retinas of *Atm*<sup>+/+</sup> and *Atm*<sup>-/-</sup> mice and quantification of the results ( $n = 4$  mice per genotype). ECs, endothelial cells. **(e)** Isolectin staining of whole-mount retinal samples of *Atm*<sup>+/+</sup> and *Atm*<sup>-/-</sup> mice at P8 and at P11 in the ischemic retinopathy model (left) and quantification of the vaso-obliterated area at P11 (right;  $n = 6$  mice per group). **(f)** Isolectin staining of whole-mount retinas of *Atm*<sup>VEC-KO</sup> and control mice at P16 in the ischemic retinopathy model and quantification of the results ( $n = 6$  mice per group). The control group in **f** and **g** were Cre-negative mice (Online Methods). **(g)** Isolectin staining of the retinas of *Atm*<sup>VEC-KO</sup> and control mice at P4. Open arrowheads indicate the area of vessel sparsity in *Atm*<sup>VEC-KO</sup> mice. **(h)** Immunocytochemistry of HUVECs to detect BrdU and TUNEL staining after treatment with the ATM inhibitor KU-55933 (KU) or DMSO and quantification of the results ( $n = 4$  mice per group). Closed arrowheads indicate cell proliferation and open arrowheads indicate cell apoptosis. **(i)** Quantification of the percentage of viable HUVECs cultured in various concentrations of KU-55933 over the indicated time course ( $n = 4$  mice per group). **(j)** Immunocytochemistry of primary lung endothelial cells from *Atm*<sup>+/+</sup> and *Atm*<sup>-/-</sup> mice to detect VE-cadherin (VE-cad), BrdU and caspase 3 (Casp3) and quantification of the results ( $n = 4$  mice per group). Closed arrowheads indicate proliferating endothelial cells, and open arrowheads indicate apoptotic endothelial cells. All data are means  $\pm$  s.e.m. Scale bars, **a–g**, 500  $\mu$ m; **d,h,j**, 50  $\mu$ m. \* $P < 0.05$ , \*\* $P < 0.01$ , NS, not significant by two-tailed Student's *t* test.

These results suggest that the reduced number of neovascular tufts in *Atm*<sup>-/-</sup> mice at P16 is a result specifically of effects on neovascular formation. At P14, *Atm* deficiency did not alter VEGF receptor expression (Supplementary Fig. 3c,d). This result suggests that *Atm* functions in angiogenesis independently of VEGF signaling.

We next investigated whether *Atm* deficiency affected other cell types in the retina. Pericytes are characterized by expression of desmin and  $\alpha$ -smooth muscle actin (ASMA). In the ischemic retinopathy model, we detected similar numbers of desmin<sup>+</sup> cells in the neovascular tufts of *Atm*<sup>-/-</sup> mice as in *Atm*<sup>+/+</sup> mice, whereas we did not detect ASMA-positive cells in the neovascular tufts of either strain (Supplementary Fig. 5a). The recruitment of myeloid cells,

including macrophages, which may affect the formation of neovascular tufts<sup>1</sup>, was also unaffected in *Atm*<sup>-/-</sup> mice (Supplementary Fig. 5b,c). Results from a recent study indicate that astrocytes in aged *Atm*<sup>-/-</sup> mice have abnormal morphology<sup>14</sup>. In this stage of our study, we did not detect immunoreactivity for pAtm in the astrocytes of *Atm*<sup>+/+</sup> mice (Supplementary Fig. 5e) and did not note any astrocytic defects in *Atm*<sup>-/-</sup> mice (Supplementary Fig. 5f–i). However, some neuroretinal cells in *Atm*<sup>+/+</sup> mice had very weak expression of pAtm (Supplementary Fig. 2c,d,g,h).

To confirm that the phenotype of *Atm*<sup>-/-</sup> mice was caused by loss of *Atm* in endothelial cells, we generated an endothelial-specific *Atm* knockout strain (*Atm*<sup>VEC-KO</sup> mice) (Supplementary Fig. 2b–d,g,h).

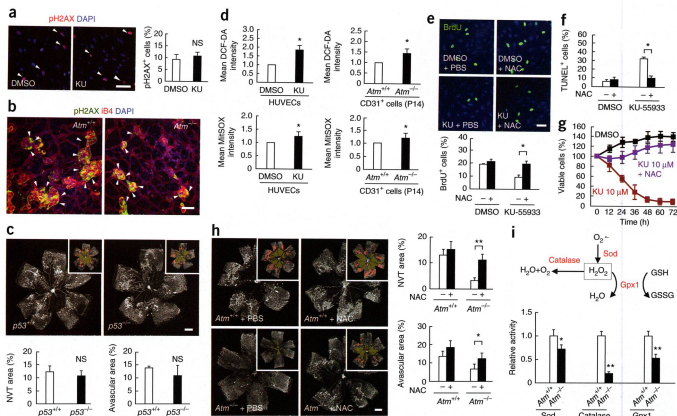


**Figure 3** *Atm* promotes endothelial proliferation by suppressing ROS accumulation. (a) Immunohistochemistry of HUVECs treated with KU-55933 (KU) or DMSO to detect pH2AX and quantification of the results (*n* = 4 independent experiments for each condition). Arrowheads indicate pH2AX-positive cells. (b) Immunohistochemistry of whole-mount retinas of *Atm*<sup>-/-</sup> and *Atm*<sup>+/-</sup> mice at P14 in the ischemic retinopathy model. Arrowheads indicate pH2AX expression in neovascular tufts. (c) Isolecithin staining of *p53*<sup>-/-</sup> and *p53*<sup>+/-</sup> whole-mount retinas at P16 in the ischemic retinopathy model and quantification of the results (*n* = 6 mice per group). (d) Quantification of DCF-DA and MitSOX staining in KU-55933-treated or DMSO-treated HUVECs and in CD31<sup>+</sup> endothelial cells isolated from *Atm*<sup>-/-</sup> or *Atm*<sup>+/-</sup> mice at P14 in the ischemic retinopathy model (*n* = 6 mice per group). (e) BrdU staining of HUVECs treated as indicated and quantification of the results (*n* = 4 independent experiments for each condition). (f, g) Quantification of the percentage of TUNEL-positive HUVECs after KU-55933 or DMSO treatment (f) and quantification of the percentage of viable HUVECs after the indicated treatments and over the indicated time course (g; *n* = 4 independent experiments per condition). (h) Isolecithin staining of whole-mount retinas of *Atm*<sup>-/-</sup> and *Atm*<sup>+/-</sup> mice treated systemically with NAC or PBS at P16 in the ischemic retinopathy model and quantification of the results (*n* = 6 mice per group). (i) Schematic diagram of the enzymatic activities of Sod, catalase and Gpx1 (top) and measurement of their activities in *Atm*<sup>-/-</sup> or *Atm*<sup>+/-</sup> endothelial cells (bottom; *n* = 4 mice per group). GSH, glutathione; GSSG, glutathione disulfide. All data are mean  $\pm$  s.e.m. Scale bars, c, h, 500  $\mu$ m; a, b, e, 50  $\mu$ m. \**P* < 0.05, \*\**P* < 0.01, NS, not significant by two-tailed Student's *t* test.

Similarly to *Atm*<sup>-/-</sup> mice, *Atm*<sup>VEC-KO</sup> mice in the ischemia retinopathy model had substantially fewer neovascular tufts but enhanced normal vessel recovery compared to control mice (Fig. 2f and Online Methods). By contrast, macrophage/neutrophil-specific (LysM-Cre mice<sup>23</sup>) or neurotensin-specific ( $\alpha$ -Cre mice<sup>24</sup>) deletion of *Atm* did not alter the ischemic retinopathy phenotype (Supplementary Fig. 5d).

We next more thoroughly examined the consequences of *Atm* deficiency on normal postnatal retinal development. In the early neonatal period (P4) in wild-type mice, pATM had very weak expression in both endothelial stalk and neuroretinal cells (Supplementary Fig. 6a). At P4, *Atm*<sup>-/-</sup> and *Atm*<sup>VEC-KO</sup> mice had a slightly lower vessel density than did their respective control mice (Fig. 2g and Supplementary Fig. 6a, b). However, as shown in Figure 2e, this minor and transient defect normalized at P8 in *Atm*<sup>-/-</sup> mice, and we detected no vascular defects in adult *Atm*<sup>-/-</sup> mice (Supplementary Fig. 6c–i).

We next tested the function of ATM *in vitro*. Exposure of cultured HUVECs to KU-55933, an ATM-kinase-specific inhibitor, markedly decreased cell proliferation, increased apoptosis and reduced the

number of viable cells in a concentration-dependent manner (Fig. 2h, i). KU-55933 did not inhibit the growth of nonendothelial cell lines, even at the highest concentration tested (10  $\mu$ M; Supplementary Fig. 7), consistent with previously published results<sup>25</sup>. Cultured primary lung endothelial cells<sup>26</sup> from *Atm*<sup>-/-</sup> mice had a reduced level of cell proliferation and more apoptosis than did cells from *Atm*<sup>+/-</sup> mice (Fig. 2j). Exposure to KU-55933 did not affect the *Atm*<sup>-/-</sup> cells, confirming the specificity of this compound (data not shown).

#### ATM promotes endothelial proliferation by suppressing ROS

ATM acts as a master regulator of the DDR by phosphorylating downstream proteins, including H2AX and p53 (ref. 9). Exposure of HUVECs to KU-55933 did not affect the  $\gamma$ -phosphorylation of H2AX (pH2AX) (Fig. 3a). Primary cultured lung *Atm*<sup>+/-</sup> and *Atm*<sup>-/-</sup> endothelial cells also showed no substantial difference in their pH2AX staining (data not shown). pH2AX staining was also not altered in the neovascular tufts of *Atm*<sup>-/-</sup> mice compared to *Atm*<sup>+/-</sup> mice (Fig. 3b). These results suggest that ATM functions in angiogenesis independently of canonical DDR signaling.

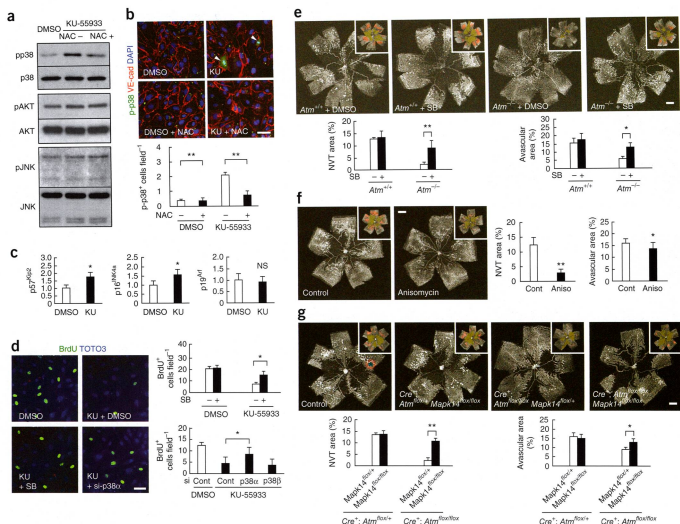


**Figure 4** p38 $\alpha$  acts downstream of *Atm* in endothelial cells. **(a)** Western blotting of HUVECs with the indicated treatments to detect the indicated proteins. **(b)** Immunocytochemistry of HUVECs after the same treatments as in **a** and quantification of the number of p-p38-positive cells ( $n = 4$  independent experiments per condition). Arrowheads indicate p-p38-positive HUVECs. KU, KU-55933; VE-cad, VE-cadherin. **(c)** Quantitative PCR analysis of the indicated CDK inhibitors in KU-55933-treated or DMSO-treated HUVECs ( $n = 4$  independent experiments per condition). **(d)** Immunocytochemistry of HUVECs after the treatments with KU-55933 or DMSO and siRNAs as indicated to detect BrdU and TOTO3 and quantification of the results ( $n = 4$  independent experiments per condition). si, siRNA targeting the indicated proteins. **(e)** Isolecithin staining of whole-mount retinas of *Atm*<sup>-/-</sup> and *Atm*<sup>+/+</sup> mice treated with the p38 kinase inhibitor SB203580 (SB) or vehicle (DMSO) as indicated at P16 in the ischemic retinopathy model and quantification of the results ( $n = 6$  mice per group). Mice were intraperitoneally treated with SB203580 or vehicle daily from P11 to P15. **(f)** Isolecithin staining of whole-mount wild-type retinas treated with the p38 activator anisomycin (Aniso) or the vehicle control at P16 in the ischemic retinopathy model and quantification of the results ( $n = 6$  mice per group). Mice were intraperitoneally treated with anisomycin or vehicle (DMSO) at P14. **(g)** Isolecithin staining of whole-mount retinas of control mice or of mice with endothelial-specific deficiency of *Atm* (*Cre*; *Atm*<sup>lox/lox</sup>) and/or *p38α* (*Cre*; *Mapk14*<sup>lox/lox</sup>) at P16 in the ischemic retinopathy model and quantification of the results ( $n = 7$  mice per group). The control group was *Cre*; *Atm*<sup>lox/lox</sup>; *Mapk14*<sup>lox/lox</sup> mice. All data are mean  $\pm$  s.e.m. Scale bars, **e–g**, 500  $\mu$ m; **b, d**, 50  $\mu$ m. \* $P < 0.05$ , \*\* $P < 0.01$ , NS, not significant by two-tailed Student's *t* test.

We next tested the response of *p53*<sup>-/-</sup> mice in the ischemic retinopathy model. The retinal vascular response was not substantially different in these mice compared to *p53*<sup>+/+</sup> control mice (Fig. 3c). These results also suggest that *Atm* functions in angiogenesis independently of canonical DDR signaling involving *p53*.

It has previously been suggested that *Atm* might contribute to oxidative defense through induction of the major antioxidant systems<sup>27–29</sup>. In line with this concept, the amounts of cytosolic ROS (detected by DCF-DA) and mitochondrial ROS (detected by MitoSOX staining) were higher in HUVECs treated with KU-55933 compared to those treated with DMSO control. Similarly, the amounts of cytosolic and

mitochondrial ROS were higher in CD31<sup>+</sup> endothelial cells in *Atm*<sup>-/-</sup> mice subjected to ischemic retinopathy compared to *Atm*<sup>+/+</sup> mice in the same model (Fig. 3d). Notably, exposure to NAC abrogated the effects of KU-55933 on HUVECs (Fig. 3e–g) and of *Atm* deficiency in primary cultured lung endothelial cells (Supplementary Fig. 8). *In vivo*, intraperitoneal administration of NAC to *Atm*<sup>-/-</sup> mice reversed the effect on *Atm* deficiency on neovascular tuft formation (Fig. 3h). These results suggest that the proangiogenic effect of *Atm* is mediated by its function in oxidative defense.

To explore which signaling cascades might be involved in *Atm*-mediated oxidative defense, we examined the activity of various



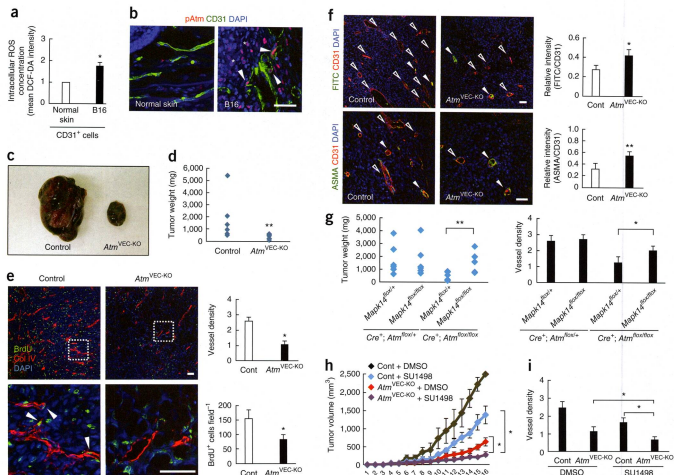


**Figure 5** Loss of *Atm* decreases tumor angiogenesis and enhances the antiangiogenic activity of *Vegf* blockade. **(a)** Quantification of DCF-DA staining in CD31<sup>+</sup> endothelial cells isolated from B16 tumors or from normal subcutaneous tissue ( $n = 5$  mice per group). **(b)** Immunohistochemistry to detect pAtm, CD31 and DAPI on in tissue sections from B16 tumors or normal subcutaneous tissue. Arrowheads indicate pAtm-positive endothelial cells in tumors, and asterisks indicate pAtm-positive tumor cells. **(c)** Macroscopic appearance of B16 tumors implanted in *Atm*<sup>+/+</sup> or control (*Cre*<sup>+</sup>) mice. **(d)** Quantification of tumor weight 10 d after transplantation ( $n = 6$  mice per group). **(e)** Immunohistochemistry to detect BrdU, collagen IV (Col IV) and DAPI in tumors 10 d after transplantation and quantification of the results ( $n = 6$  mice per group). The boxes in the images in the top row indicate areas of high magnification shown in the images in the bottom row. Arrowheads indicate BrdU-positive endothelial cells. **(f)** Immunohistochemistry to detect FITC, CD31, ASMA and DAPI in tumors of mice injected intracardially with FITC-dextran (left) and quantification of the results (right;  $n = 5$  mice per group). Open arrowheads indicate immature vessels (lacking blood flow or ASMA-positive pericytes) and closed arrowheads indicate mature vessels (with blood flow or ASMA-positive pericytes). **(g)** Quantification of tumor weight and vessel density 10 d after transplantation in mice with endothelial-specific deficiency of *Atm* and/or *p38* ( $n = 4-6$  in each genotype). **(h)** Quantification of tumor volume in tumors of control or *Atm*<sup>VEC-KO</sup> mice treated with DMSO or the *Vegf*2 inhibitor SU1498 as indicated ( $n = 5$  mice per group). The values on the x axis indicate days after transplantation. **(i)** Quantification of tumor vessel density in the same tumors as in **h** at 10 d after transplantation. In **e**, **g** and **i**, vessel density is shown as the qualitative intensity of collagen IV staining. All data are mean  $\pm$  s.e.m. Scale bars, 50  $\mu$ m. \* $P < 0.05$ , \*\* $P < 0.01$  by two-tailed Student's *t* test.

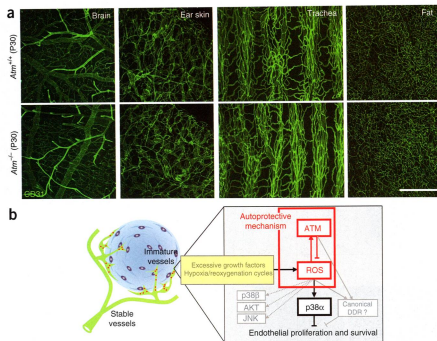
enzymes involved in ROS degradation. Compared to *Atm*<sup>+/+</sup> cells, *Atm*<sup>-/-</sup> endothelial cells had markedly lower activity of two of these enzymes, glutathione peroxidase 1 (Gpx1) and catalase (Fig. 3i), which convert cytosolic hydrogen peroxide to water or to oxygen plus water<sup>30</sup>. The activity of superoxide dismutase (Sod), which converts mitochondrial superoxide into hydrogen peroxide, was also lower in *Atm*<sup>-/-</sup> than in *Atm*<sup>+/+</sup> endothelial cells (Fig. 3i). As the effect on catalase activity was especially prominent, we intraocularly delivered an adenovirus coding for catalase. Catalase overexpression in *Atm*<sup>-/-</sup> mice resulted in an increase in the number of neovascular tufts and in the avascular area, although not to the levels seen in *Atm*<sup>+/+</sup> mice (Supplementary Fig. 9a-f). Conversely, treatment of wild-type mice with an inhibitor of Gpx1, mercaptosuccinate, resulted in a modest decrease in the number of neovascular tufts and in the avascular area (Supplementary Fig. 9g,h).

### p38 $\alpha$ acts downstream of ATM in endothelial cells

We next examined how ATM inhibition or deficiency affected stress-induced intracellular pathways. Treatment of HUVECs with KU-55933 led to increased p38 phosphorylation, indicating its activation. This activation was suppressed by the simultaneous administration of NAC, suggesting that ATM inhibition activates p38 by increasing the amount of ROS (Fig. 4a,b). In contrast, KU-55933 exposure did not affect AKT or JNK phosphorylation (Fig. 4a).

We next investigated the activation of various kinases involved in the canonical DDR pathway. Although phosphorylation of CHK2, a direct target of the ATM kinase, was decreased in HUVECs treated with KU-55933, phosphorylation of ATR, p53 or CHK1 was not affected (Supplementary Fig. 10a). Moreover, ATM inhibition did not cause DNA damage in HUVECs, as measured by a comet

**Figure 6** Atm deficiency does not affect the maintenance of healthy vessels in adult mice. **(a)** Whole-mount immunohistochemistry of CD31 in the indicated tissues of *Atm*<sup>-/-</sup> and *Atm*<sup>+/+</sup> mice at P30. **(b)** Proposed model for the selective activation of ATM in immature vessels and for the proangiogenic function of ATM through decreasing oxidative stress and p38 $\alpha$  activation. Scale bar, 500  $\mu$ m.



assay (Supplementary Fig. 10b,c). These results suggest that the ATR-CHK1 pathway compensates for the lack of ATM activity in the DDR, as that cascade may act as a parallel pathway in the DDR. ATM inhibition in HUVECs did not affect the expression or activation of other possible targets of ATM, such as AMP-activated protein kinase (AMPK), STAT3 or HIF-1 $\alpha$ <sup>10,31</sup> (Supplementary Fig. 10d).

p38 $\alpha$ , the most prominent isoform of p38, induces growth arrest and apoptosis through the upregulation of cyclin-dependent kinase (CDK) inhibitors and apoptosis-related genes<sup>32,33</sup>. KU-55933-treated HUVECs had higher expression of CDK inhibitors and proapoptotic genes than did DMSO-treated control cells (Fig. 4c and Supplementary Fig. 11a). Intraperitoneal treatment of *Atm*<sup>VEC-KO</sup> mice with the broad-spectrum caspase inhibitor acetyl-TyrValAlaAsp chloromethyl ketone (YVAD-cmk) had an increased number of neovascular tufts and an increased amount of vascular recovery, although not to the levels of *Atm*-intact control mice (Supplementary Fig. 11b–d). These data suggest that the effects of ATM inhibition or deficiency might be mediated through p38 $\alpha$  activation. Consistent with this idea, administration of an inhibitor of p38 kinase activity (SB203580) or of a p38 $\alpha$ -targeted siRNA, but not of a p38 $\beta$ -targeted siRNA, abrogated the growth inhibitory effects of KU-55933 in HUVECs (Fig. 4d).

We next tested the effects of ROS on p38 activation. Treatment of HUVECs with hydrogen peroxide increased p38 activation (Supplementary Fig. 12a). p38 was activated in the neovascular tufts of *Atm*<sup>-/-</sup> mice *in vivo*, an effect that was abolished by treatment with NAC (Supplementary Fig. 12b,c). Intraperitoneal administration of SB203580 reversed the effect of *Atm* deficiency on neovascular tuft formation (Fig. 4e). These data suggest that excessive, ROS-mediated activation of p38 underlies the vascular phenotype of *Atm*<sup>-/-</sup> mice. To test this hypothesis further, we treated wild-type mice with anisomycin, a p38 activator. Anisomycin treatment largely mimicked the vascular phenotype of the *Atm*<sup>-/-</sup> mice (Fig. 4f). The effect of anisomycin was equivalent to that of *Atm* deletion on neovascular tufts, however, the effect of anisomycin was smaller than that of *Atm* deletion on the avascular area. As anisomycin is a JNK activator as well as a p38 activator, we used mice lacking p38 $\alpha$  to test the requirement of p38 $\alpha$  in mediating anisomycin's effects. Endothelial-specific p38 $\alpha$  deficiency did not have an effect on the vasculature in the ischemic retinopathy model (Supplementary Fig. 13), and anisomycin did not affect the phenotype of these mice (data not shown), consistent with the idea that p38 activation mediates anisomycin's effects.

To reinforce the link between *Atm* and p38 $\alpha$ , we generated mice with an endothelial-specific deficiency of both *Atm* and p38 $\alpha$ . These mice had an increased number of neovascular tufts and less

normal vascular recovery compared to *Atm*<sup>VEC-KO</sup> mice (Fig. 4g). However, rescue of the *Atm* phenotype by p38 $\alpha$  deficiency was only partial, indicating that other compensatory mechanisms may be involved downstream of the ATM-ROS pathway.

### Loss of *Atm* in endothelium decreases tumor angiogenesis

We next tested whether the proangiogenic function of *Atm* would have relevance to tumor angiogenesis. We implanted mice with B16 melanoma cells subcutaneously. Using DCF-DA staining, we found that endothelial cells isolated from these tumors had higher concentrations of ROS than did endothelial cells isolated from normal subcutaneous tissue (Fig. 5a). We detected an abundance of pAtm in the endothelial cells of tumor vessels but not in those of stable subcutaneous vessels (Fig. 5b). Tumor growth and tumor vessel density were lower in *Atm*<sup>VEC-KO</sup> mice than in control mice (Fig. 5c–e and Online Methods). *Atm*<sup>VEC-KO</sup> mice had fewer BrdU-positive endothelial cells and BrdU-positive tumor cells than did control mice (Fig. 5e). The number of immature vessels, characterized by poor blood flow and insufficient coverage with ASMA-positive pericytes<sup>4,18</sup>, was markedly lower in *Atm*<sup>VEC-KO</sup> mice than in control mice (Fig. 5f). These findings are consistent with the phenotype of *Atm*<sup>VEC-KO</sup> mice in the ischemic retinopathy model, in which the vessels of the neovascular tufts are immature, have relatively poor perfusion<sup>19</sup> and lack ASMA-positive pericytes (Supplementary Fig. 5a). As in the ischemic retinopathy model, *Atm*<sup>VEC-KO</sup> mice with endothelial-specific p38 $\alpha$  deficiency had a larger tumor volume and significantly more vascularization than did *Atm*<sup>VEC-KO</sup> mice (Fig. 5g and Supplementary Fig. 14).

The poor response to anti-VEGF therapy observed in some human patients is partly a result of VEGF-independent proangiogenic mechanisms<sup>5</sup>. As ATM might mediate one such mechanism, we tested whether *Atm* deficiency would enhance the antiangiogenic effect of Vegf inhibition in mice. Vegf inhibition by SU1498, a potent and selective inhibitor of Vegfr2, and *Atm* deficiency had synergistic effects in inhibiting tumor growth and angiogenesis in the B16 tumor model (Fig. 5h,i).

We next examined the effect of Atm deficiency in normal healthy vessels. In contrast to the effects of Atm deficiency on pathological retinal and tumor angiogenesis, normal vessels in a number of different types of tissue examined in adult mice were not affected by Atm deficiency (Fig. 6a).

## DISCUSSION

Here we have shown that ROS substantially accumulates in newly formed immature vessels and activates Atm. Loss of Atm in endothelial cells minimizes pathological ocular and tumor neoangiogenesis as a consequence of defective oxidative defense rather than an impaired DDR (Fig. 6b).

A recent study reported that a major DDR protein, H2AX, is integral to pathological neoangiogenesis<sup>11</sup>. In that study, silencing ATR, but not ATM, substantially decreased the  $\gamma$ -phosphorylation of H2AX, suggesting that DDR signaling is dependent on ATR rather than ATM in endothelial cells. In agreement with those findings, our data indicate that the major role of Atm in endothelial cells is in oxidative defense rather than in the H2AX-mediated DDR pathway. Another previous report indicated that ATM promotes senescence in endothelial cells through activation of the DDR pathway<sup>12</sup>, suggesting that ATM may regulate endothelial senescence through the DDR and angiogenesis through its role in oxidative defense.

Various biological processes may be involved in ROS production in immature vessels associated with pathological angiogenesis. Chromosomal abnormalities in the tumor endothelium have been shown to be causative in the production of ROS<sup>34</sup>. Exposure to excessive growth factors results in cell proliferation and induces intracellular ROS production<sup>35</sup>. Furthermore, repeated cycles of hypoxia and reoxygenation result in the production of ROS within tissues or cells<sup>36</sup>. Indeed, we found that in HUVECs, hypoxia-reoxygenation cycles markedly increased ATM activation.

The contribution of ATM to oxidative defense involves multiple antioxidant proteins, including catalase, Gpx, Sod and glucose-6-phosphate dehydrogenase (G6PD)<sup>28,29,37</sup>. Although we showed that catalase has an important role in this context, it is difficult to determine which antioxidant protein(s) are crucial, as they function to degrade ROS in a complementary manner<sup>30</sup>. Previous reports have shown that oxidative stress promotes neoangiogenesis and worsens the severity of neovascular disease in a Vegf- and Tlr2-dependent manner<sup>38–40</sup>. Taken together with our findings, ROS could be viewed as 'a double-edged sword' in cancer therapy: whereas tumor angiogenesis seems to be stimulated by a certain amount of ROS, excessive amounts of ROS, as in the case of Atm deficiency, seems to be suppressive. Further investigation is needed to determine which ROS-related pathways are proangiogenic and which are antiangiogenic.

Our findings indicate that agents targeting ATM could potentially enhance the antiangiogenic efficacy of VEGF blockade. As ATM inhibition enhances the radiation sensitivity of cancer cells<sup>10</sup>, ATM targeting might have effects not only in suppressing tumor angiogenesis but also in enhancing the antitumor activity of radiation therapy. However, the DDR is a major barrier against tumorigenesis, and the loss of ATM could enhance the mutagenic effects of chemotherapeutic strategies. Therefore, a drug that would specifically target ATM-mediated oxidative defense might be more effective than an ATM kinase inhibitor. In this respect, targeting the cysteine residue at position 2991 of ATM, crucial to its activation by oxidative stress<sup>41</sup>, may be a potential therapeutic strategy.

## METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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## AUTHOR CONTRIBUTIONS

Y.O. and A.N.-I. performed experiments and analyzed data. T.S. interpreted results and assisted in manuscript preparation. K.O. provided experimental materials. Y.K. designed experiments, interpreted results and wrote the paper.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# The pulmonary endothelial glycocalyx regulates neutrophil adhesion and lung injury during experimental sepsis

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Sepsis, a systemic inflammatory response to infection, commonly progresses to acute lung injury (ALI), an inflammatory lung disease with high morbidity. We postulated that sepsis-associated ALI is initiated by degradation of the pulmonary endothelial glycocalyx, leading to neutrophil adherence and inflammation. Using intravital microscopy, we found that endotoxemia in mice rapidly induced pulmonary microvascular glycocalyx degradation via tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )-dependent mechanisms. Glycocalyx degradation involved the specific loss of heparan sulfate and coincided with activation of endothelial heparanase, a TNF- $\alpha$ -responsive, heparan sulfate-specific glucuronidase. Glycocalyx degradation increased the availability of endothelial surface adhesion molecules to circulating microspheres and contributed to neutrophil adhesion. Heparanase inhibition prevented endotoxemia-associated glycocalyx loss and neutrophil adhesion and, accordingly, attenuated sepsis-induced ALI and mortality in mice. These findings are potentially relevant to human disease, as sepsis-associated respiratory failure in humans was associated with higher plasma heparan sulfate degradation activity; moreover, heparanase content was higher in human lung biopsies showing diffuse alveolar damage than in normal human lung tissue.

Sepsis is a common and severe medical condition characterized by a systemic inflammatory response to infection<sup>1</sup>. The inflammatory consequences of sepsis are particularly apparent within the pulmonary circulation, which is continuously exposed to circulating pathogen-associated molecular patterns (such as the endotoxin lipopolysaccharide (LPS)) capable of triggering innate immunity. Over 40% of individuals with sepsis develop ALI, a syndrome characterized by neutrophilic inflammation and pulmonary vascular hyperpermeability<sup>2</sup>. The development of ALI markedly worsens patient prognosis, increasing intensive care unit mortality from 11% to 38% in patients with septic shock<sup>3</sup>. Survivors of ALI are often left with considerable long-term morbidity and increased healthcare expenditures<sup>4</sup>. Despite this clinical significance, no sepsis-specific treatments prevent the onset of inflammatory lung injury, reflecting an incomplete understanding of septic ALI pathogenesis.

Neutrophil adhesion to the vascular intima is crucial to the initiation of inflammatory tissue injury. Intimal surfaces are lined by the endothelial glycocalyx, an extracellular layer of glycoproteins, proteoglycans and glycosaminoglycans (GAGs)<sup>5</sup>. *In vivo*, heparan sulfate and other glycocalyx GAGs are highly hydrated, forming a substantial endothelial surface layer (ESL) that acts as a barrier to circulating cells and large molecules (Fig. 1a)<sup>6</sup>. Little, however, is known about the influence of the glycocalyx on neutrophil adhesion during inflammation<sup>7</sup>.

Enzymatic degradation of the glycocalyx in isolated guinea pig coronary capillaries, microperfused rat mesenteric venules and microperfused mouse cremasteric venules is associated with increased leukocyte retention; however, the mechanisms underlying this association are uncertain<sup>7–10</sup>. Glycocalyx degradation-associated neutrophil retention may be relevant to sepsis-induced organ inflammation, given recent observations of circulating glycocalyx fragments in patients with septic shock<sup>11</sup>.

Our study aimed to elucidate the mechanisms by which glycocalyx loss occurs during sepsis and how this loss allows for neutrophil adhesion within the pulmonary circulation. We hypothesized that sepsis induces activation of heparanase (an endogenous heparan sulfate-specific glucuronidase) within the pulmonary microvasculature, with consequent degradation of the pulmonary endothelial glycocalyx playing a central part in neutrophil adherence and inflammatory lung injury. Our findings may suggest new therapeutic approaches to prevent the onset of inflammatory lung injury during sepsis.

## RESULTS

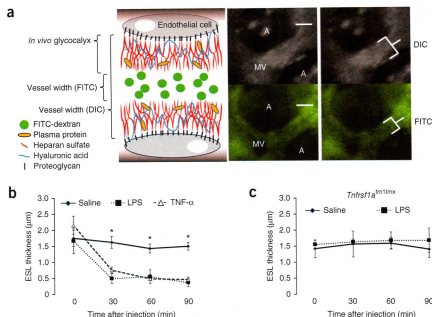
### Endotoxemia induces ESL loss via activation of heparanase

Intravital microscopy (*in vivo* microscopy, IVM) of the ESL provides an ideal means of studying glycocalyx structure during disease states, as the glycocalyx has been found to be aberrant when studied

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**Figure 1** LPS degrades the pulmonary ESL via TNF- $\alpha$ . (a) Left, graphical representation (not to scale) of the *in vivo* endothelial glycocalyx, forming a substantial ESL that excludes large molecules (for example, dextrans) from the vessel surface. Right, representative images of mouse subpleural microvessels (MV) using simultaneous differential interference contrast (DIC) and FITC-dextran (FITC) microscopy. Differences in DIC and FITC vascular widths (inclusive and exclusive of the ESL, respectively) reflect ESL thickness (brackets). Scale bars, 10  $\mu$ m. A, alveolus. (b) Assessment of pulmonary ESL thickness within subpleural microvessels (mean DIC diameter  $18.11 \pm 1.01$   $\mu$ m, mean FITC diameter  $14.17 \pm 0.96$   $\mu$ m) of wild-type mice injected with intravenous saline, LPS (20  $\mu$ g per g body weight) or TNF- $\alpha$  (200 ng) at  $t = 0$  min and imaged at 0, 30, 60 and 90 min.  $n = 5$  mice per group;  $*P < 0.05$  in comparison to other groups. (c) Assessment of pulmonary ESL thickness of TNFR1-deficient *Tnfrsf1<sup>tm1Lmx</sup>* mice treated at  $t = 0$  min with intravenous saline or LPS (20  $\mu$ g per g body weight);  $n = 3$  or 4 mice per group. Data are represented as means  $\pm$  s.e.m.



using *in vitro* preparations and is often damaged during tissue handling and fixation<sup>5,12,13</sup>. We therefore used closed-chest mouse pulmonary IVM to determine glycocalyx integrity by measuring ESL exclusion of fluorescently labeled, high-molecular-weight (150 kDa) dextrans from vessel surfaces (Fig. 1a and Supplementary Fig. 1). The mean baseline pulmonary microvascular ESL thickness of the wild-type C57BL/6 mice studied ( $n = 43$ ) was  $1.67 \pm 0.09$   $\mu$ m (mean  $\pm$  s.e.m.), substantially greater than the ESL thickness observed in cremasteric (systemic) microvessels ( $0.67 \pm 0.08$   $\mu$ m,  $n = 9$ , Supplementary Fig. 2a). In saline-treated mice, pulmonary ESL thickness remained stable throughout 90 min of observation (Fig. 1b). Intravenous injection of LPS (20  $\mu$ g per g body weight) induced a rapid (<30 min) loss of pulmonary ESL thickness, suggesting sepsis-associated glycocalyx degradation (Fig. 1b). Intravenous injection of TNF- $\alpha$ , a cytokine released early in sepsis, similarly triggered rapid pulmonary ESL loss (Fig. 1b). LPS-induced pulmonary ESL loss was prevented in mice lacking TNFR1, the major TNF- $\alpha$  receptor implicated in sepsis<sup>14</sup>, suggesting a necessary role for TNF- $\alpha$  in septic glycocalyx degradation (Fig. 1c). Notably, cremasteric ESL thickness was unchanged after LPS injection, suggesting vascular bed heterogeneity in the response to endotoxemia (Supplementary Fig. 2a).

Intravenous injection of heparinase-III, a heparan sulfate-specific bacterial glucuronidase<sup>15</sup>, rapidly decreased pulmonary ESL thickness in wild-type mice, demonstrating the essential role of glycocalyx heparan sulfate in the maintenance of ESL structure (Fig. 2a). We hypothesized that LPS, via TNF- $\alpha$ -dependent mechanisms, similarly induces the degradation of the pulmonary ESL by rapidly activating pulmonary endothelial heparanase, a mammalian heparinase-III analog constitutively expressed as a 65-kDa proenzyme<sup>16</sup>. Treatment of mouse lung microvascular endothelial cells (MLMVECs) with TNF- $\alpha$  rapidly (within 30 min) induced cleavage of 65-kDa heparanase to its active 50-kDa isoform (Fig. 2b), coincident with increased heparan sulfate degradation activity within cell lysates (Fig. 2c).

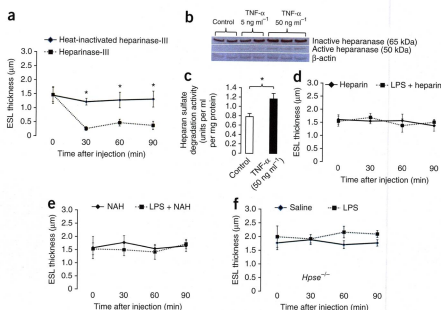
*In vivo* inhibition of heparanase activity with the competitive antagonist heparin completely prevented endotoxemia-induced ESL loss (Fig. 2d) without interfering with LPS activation of Toll-like receptor

signaling (Supplementary Fig. 3). This effect of heparin was independent of anticoagulation, as ESL loss was also prevented by the nonanticoagulant heparanase inhibitor *N*-desulfated/re-*N*-acetylated heparin (NAH, Fig. 2e) as well as in mice genetically lacking heparanase expression (*Hps<sup>-/-</sup>* mice, Fig. 2f). These findings demonstrate that activation of constitutively expressed endothelial heparanase mediates the rapid thinning of the pulmonary ESL that occurs after LPS exposure.

### The pulmonary ESL regulates neutrophil adhesion

To assess in real time the relationship between ESL loss and pulmonary neutrophil recruitment, a key early step in the alveolar inflammation characteristic of ALI, we performed adoptive transfer of GFP-expressing neutrophils into wild-type mice. Circulating GFP<sup>+</sup> neutrophils adhered to the pulmonary endothelium within 30 min of intravenous LPS administration (remaining stationary for >15 min thereafter, Fig. 3a and Supplementary Fig. 4), a time course that closely followed ESL loss (Figs. 1b and 2a). Similarly, fluorescent microspheres labeled with antibodies against intercellular adhesion molecule 1 (ICAM-1), an endothelial adhesion molecule implicated in endotoxin-induced pulmonary neutrophil adhesion (Supplementary Fig. 5) and lung injury<sup>17</sup>, bound to microvascular endothelial surfaces within 45 min of LPS administration (Fig. 3b). These findings were not limited to the subpleural microvasculature, as we noted concordant microsphere adhesion throughout frozen sections of the left lung (Supplementary Fig. 6). The possibility that anti-ICAM-1 microspheres were being captured by neutrophils was excluded, as neutrophil depletion did not prevent microsphere adhesion during endotoxemia (Supplementary Fig. 7). As other adhesion molecules in addition to ICAM-1 participate in neutrophil-endothelial adhesion<sup>18</sup>, we repeated our experiments with microspheres targeted to vascular cell adhesion molecule 1 (VCAM-1); these similarly adhered to the microvasculature 45 min after LPS administration (Supplementary Fig. 8a). Together, these findings suggest that the rapid neutrophil-endothelial binding that occurs after intravenous LPS may reflect an acute change in the availability of multiple endothelial adhesion molecules to circulating cells.

**Figure 2** Heparanase mediates LPS-induced ESL degradation. (a) Assessment of pulmonary ESL thickness within subpleural microvessels of wild-type mice treated with heparanase-III or heat-inactivated heparanase-III (1 U) at  $t = 0$ ,  $n = 4$ –6 mice per group;  $*P < 0.05$ . (b,c) Expression of active (50 kDa) and inactive (65 kDa) endothelial heparanase (b, representative of three independent experiments) and heparan sulfate degradation activity (c,  $n = 4$  per group) in cultured mouse lung microvascular endothelial cells treated with TNF- $\alpha$  (5 and 50 ng ml $^{-1}$ ) or saline control for 30 min;  $*P < 0.05$ . (d) Assessment of pulmonary ESL thickness within subpleural microvessels of wild-type mice injected with intravenous vehicle or LPS (20  $\mu$ g per g body weight) in addition to the heparanase inhibitor heparin (5 U administered intravenously at  $t = 0$ );  $n = 4$ –6 mice per group. (e) Assessment of pulmonary ESL thickness within subpleural microvessels of wild-type mice injected with intravenous vehicle or LPS (20  $\mu$ g per g body weight) in addition to NAH (150  $\mu$ g administered intravenously at  $t = 0$ );  $n = 4$  or 5 mice per group. (f) Assessment of pulmonary microvascular ESL thickness in *Hps $^{-/-}$*  mice injected with intravenous saline or LPS (20  $\mu$ g per g body weight);  $n = 3$  mice per group. Data are represented as means  $\pm$  s.e.m.



To determine the mechanism by which endothelial adhesion molecule availability rapidly increases during endotoxemia, we subjected wild-type mice and MLMVECs to a short-term (45-min) LPS exposure. This exposure induced an early increase in ICAM-1 mRNA levels (Supplementary Fig. 9a,b); however, the duration of exposure was insufficient to alter ICAM-1 (or VCAM-1) total protein content or cell-surface expression (Supplementary Figs. 8b,c and 9c,d). We suspected that the increased adhesion molecule availability was instead a consequence of glycocalyx degradation, exposing constitutively expressed endothelial surface proteins typically buried within the thick pulmonary ESL<sup>10</sup>. Indeed, LPS-induced anti-ICAM-1 microsphere adhesion in the lung was attenuated by heparanase inhibition (Fig. 3b

and Supplementary Fig. 6). Furthermore, heparanase-III-induced glycolytic degradation led to anti-ICAM-1 microsphere adhesion (Fig. 3b), albeit in a heterogeneous pattern that may reflect differences between the exogenous administration of heparanase analogs and the endogenous induction of endothelial heparanase. Although heparin and heparanase-III influenced anti-ICAM-1 microsphere adhesion, they did not alter ICAM-1 mRNA levels (Supplementary Fig. 9a,b), further indicating that the rapid increase in ICAM-1 availability occurs independently of any induction of endothelial ICAM-1 expression. Finally, heparin treatment attenuated GFP $^{+}$  neutrophil adhesion after LPS exposure (Fig. 3a), consistent with previously described effects of heparin on neutrophil-endothelial cell binding in response to LPS<sup>19</sup>.

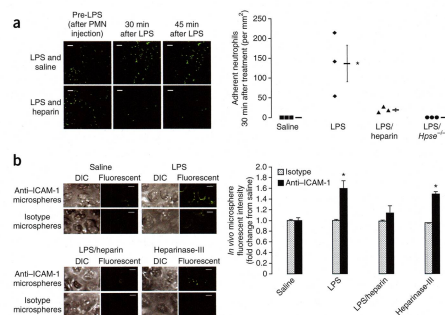


**Figure 3** LPS-induced neutrophil adherence is dependent upon ESL degradation. (a) Adherence of adoptively transferred GFP $^{+}$  neutrophils within subpleural microvessels before and 30–45 min after intravenous saline, LPS (20  $\mu$ g per g body weight) or LPS (20  $\mu$ g per g body weight) with heparin (5 U) in wild-type mice or 30–45 min after intravenous LPS (20  $\mu$ g per g body weight) in *Hps $^{-/-}$*  mice. Representative images reflect changes occurring within a single, serially imaged, low-powered field. Scale bars, 40  $\mu$ m.  $n = 3$  mice per group;  $*P < 0.05$  compared to saline. PMN, polymorphonuclear leukocytes. (b) Visualization of anti-ICAM-1-coated fluorescent microspheres within wild-type mouse subpleural microvessels, simultaneously imaged by DIC and fluorescence microscopy. Images obtained 45 min after intravenous saline, LPS (20  $\mu$ g per g body weight), LPS (20  $\mu$ g per g body weight) with heparin (5 U) or heparanase-III (1 U). Isotype-matched antibody-coated microspheres serve as controls for nonspecific adhesion. Scale bars, 20  $\mu$ m.  $n = 3$  or 4 mice per group;  $*P < 0.05$  compared to saline. Data are represented as means  $\pm$  s.e.m.

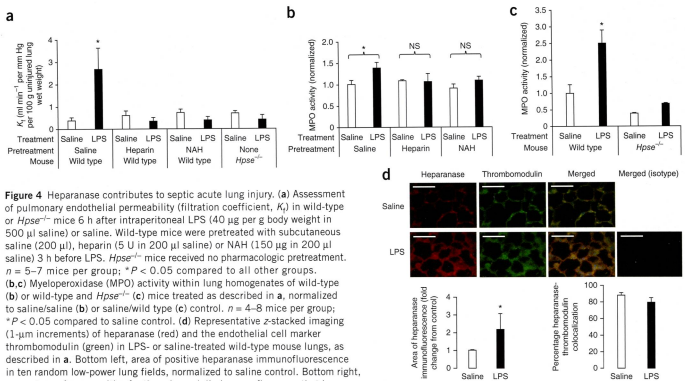


**Figure 4** Heparanase contributes to septic acute lung injury. **(a)** Assessment of pulmonary endothelial permeability (filtration coefficient,  $K_f$ ) in wild-type or *Hps*<sup>-/-</sup> mice 6 h after intraperitoneal LPS (40  $\mu$ g per g body weight in 500  $\mu$ l saline) or saline. Wild-type mice were pretreated with subcutaneous saline (200  $\mu$ l), heparin (5 U in 200  $\mu$ l saline) or NAH (150  $\mu$ g in 200  $\mu$ l saline) 3 h before LPS. *Hps*<sup>-/-</sup> mice received no pharmacologic pretreatment.  $n = 5-7$  mice per group; \* $P < 0.05$  compared to all other groups. **(b,c)** Myeloperoxidase (MPO) activity within lung homogenates of wild-type **(b)** or wild-type and *Hps*<sup>-/-</sup> **(c)** mice treated as described in **a**, normalized to saline/saline **(b)** or saline/wild type **(c)** control.  $n = 4-8$  mice per group; \* $P < 0.05$  compared to saline control. **(d)** Representative z-stacked imaging (1- $\mu$ m increments) of heparanase (red) and the endothelial cell marker thrombomodulin (green) in LPS- or saline-treated wild-type mouse lungs, as described in **a**. Bottom left, area of positive heparanase immunofluorescence in ten random low-power lung fields, normalized to saline control. Bottom right, percentage of area positive for thrombomodulin immunofluorescence that is additionally positive for heparanase, as quantified in ten random low-power fields. Scale bars, 50  $\mu$ m.  $n = 5$  mice per group; \* $P < 0.05$ . Data are represented as means  $\pm$  s.e.m.

Similarly, GFP<sup>+</sup> (*Hps*<sup>+/-</sup>) neutrophils did not adhere to the pulmonary microvasculature of LPS-treated *Hps*<sup>-/-</sup> mice (Fig. 3a). These findings provide a teleological rationale for LPS-induced heparanase activation: pathogen-associated molecular patterns prompt endothelial cells to cleave the endothelial glycocalyx, preparing the vascular surface for neutrophil adhesion and subsequent inflammation.

#### Heparanase-mediated ESL loss is necessary for septic ALI

To determine the impact of ESL loss and consequent neutrophil adhesion on the development of septic ALI, we induced ALI in wild-type mice via intraperitoneal LPS injection (40  $\mu$ g per g body weight)<sup>20</sup>. We chose this higher dose of LPS to rapidly produce robust lung injury<sup>21,22</sup>. Six hours after LPS administration, pulmonary endothelial permeability and neutrophil extravasation increased, suggestive of early ALI (Fig. 4a-c). Heparanase expression increased and was localized to the pulmonary endothelium, as demonstrated by association with the endothelial cell-surface marker thrombomodulin (Fig. 4d and Supplementary Fig. 10). Consistent with heparanase activation, small pulmonary arteries of LPS-treated mice (prepared using glycocalyx-preserving fixation techniques) had diminished vascular surface heparan sulfate content in comparison to saline-treated controls (Supplementary Fig. 11). Other glycocalyx components were unchanged, demonstrating the heparan sulfate-specific nature of heparanase activity (Supplementary Fig. 11a). Heparanase inhibitors significantly prevented LPS-induced heparan sulfate loss, endothelial hyperpermeability and neutrophil extravasation, suggesting that heparanase-mediated glycocalyx loss contributes to the later development of septic ALI (Fig. 4a,b and Supplementary Fig. 11b). Similarly, pulmonary endothelial hyperpermeability and neutrophil extravasation 6 h after intraperitoneal LPS administration were attenuated in *Hps*<sup>-/-</sup> mice compared to wild-type controls (Fig. 4a,c). Notably, lung myeloperoxidase activity was suppressed in saline-treated

*Hps*<sup>-/-</sup> mice (Fig. 4c) despite normal plasma neutrophil counts (254  $\pm$  53 neutrophils per  $\mu$ l in *Hps*<sup>-/-</sup> mice versus 197  $\pm$  47 neutrophils per  $\mu$ l in wild-type mice,  $n = 4$  per group), suggesting that heparanase influences baseline neutrophil extravasation into healthy mouse lungs. In sum, these findings demonstrate that heparanase activation (with consequent glycocalyx degradation) is necessary to the development of ALI pathophysiology during endotoxemia.

#### Heparanase in human sepsis and ALI

To determine the relevance of endothelial heparanase activation and consequent heparan sulfate degradation to human sepsis, we collected plasma samples from healthy human donors as well as from three groups of individuals with respiratory failure: those mechanically ventilated for altered mental status (for example, ischemic stroke or alcohol withdrawal), for pneumonia (that is, direct injury to the lung) and those ventilated with a primary diagnosis of nonpulmonary sepsis (that is, potentially indirect injury to the lung) (Supplementary Table 1). Plasma heparan sulfate degradation activity was elevated only in individuals with nonpulmonary sepsis, consistent with endothelial heparanase activation and release into the circulation (Fig. 5a).

We explored the relevance of heparanase to human inflammatory lung disease using human lung biopsies that showed diffuse alveolar damage, the histologic manifestation of ALI (Supplementary Table 2). These biopsies are infrequently performed, as the diagnosis of ALI during sepsis is typically made on clinical criteria alone. In normal human lung tissue, expression of heparanase was minimal (Fig. 5b,c). In biopsies with diffuse alveolar damage, heparanase immunofluorescence was eightfold higher (Fig. 5b) and was noted around capillaries and conduit vessels (Fig. 5c). These findings, although limited by a small sample size (19 plasma samples, 14 lung samples), suggest that heparanase is active in human sepsis and contributes to inflammatory lung injury.



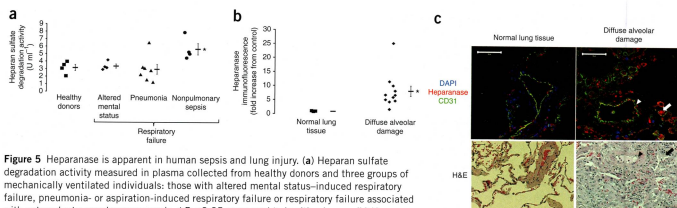


**Figure 5** Heparanase is apparent in human sepsis and lung injury. **(a)** Heparan sulfate degradation activity measured in plasma collected from healthy donors and three groups of mechanically ventilated individuals: those with altered mental status–induced respiratory failure, pneumonia– or aspiration-induced respiratory failure or respiratory failure associated with antecedent nonpulmonary sepsis.  $^*P < 0.05$  compared to healthy donors. **(b)** Heparanase immunofluorescence in normal human lung tissue and in lung biopsies with diffuse alveolar damage.  $^*P < 0.05$  compared to normal lung tissue. **(c)** Top, confocal fluorescent images of normal human lung showing minimal heparanase expression. Representative fluorescent images of a lung from a patient with diffuse alveolar damage, with high heparanase expression (red) within capillaries (arrow) and conduit vessels (arrowhead), ascertained by the endothelial marker CD31 (green). Nuclei stained with DAPI (blue). Bottom, H&E staining, with capillaries (arrow) and conduit vessels (arrowhead) noted. Scale bars, 50  $\mu$ m. Data are represented as means  $\pm$  s.e.m.

### Heparanase inhibition is protective after sepsis onset

Given that heparan sulfate degradation activity was elevated in human plasma collected a mean of 35.4 h after the onset of sepsis-associated respiratory failure (Supplementary Table 1), we hypothesized that interventions targeting heparanase activity would be lung-protective even if administered after sepsis onset. Accordingly, administration of heparin 3 h after intraperitoneal LPS administration prevented endotoxemia-induced pulmonary endothelial hyperpermeability (Fig. 6a).

We additionally tested heparin treatment in mice subjected to cecal ligation and puncture (CLP), a clinically relevant model of polymicrobial sepsis. Although CLP alone does not typically cause robust histologic lung injury, it has been demonstrated to induce endothelial and epithelial pathophysiology consistent with ALI<sup>23</sup>.

Within 24 h of CLP, mice appeared systemically ill with piloerection and decreased activity. Pulmonary heparanase expression peaked 48 h after CLP, coincident with an increase in endothelial permeability (Fig. 6b,c and Supplementary Fig. 2c). Notably, we observed no CLP-associated heparanase induction in the cremaster, a tissue with stable ESL thickness during endotoxemia (Supplementary Fig. 2). Delayed heparanase inhibition via a one-time dose of heparin (administered 24 h after CLP) attenuated pulmonary endothelial hyperpermeability (Fig. 6c), suggesting that heparin is a lung-protective intervention even in established sepsis. To augment CLP-induced neutrophilic alveolitis, we repeated CLP experiments in the presence of 60% fraction of inspired oxygen ( $\text{FiO}_2$ ), a moderate level of hyperoxia not injurious to normal mice<sup>24</sup>. In these mice, pulmonary neutrophilic infiltration was apparent 48 h after CLP (Fig. 6d).

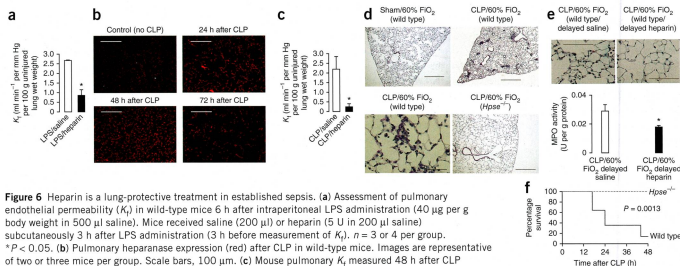


**Figure 6** Heparin is a lung-protective treatment in established sepsis. **(a)** Assessment of pulmonary endothelial permeability ( $K_p$ ) in wild-type mice 6 h after intraperitoneal LPS administration (40  $\mu$ g per g body weight in 500  $\mu$ l saline). Mice received saline (200  $\mu$ l) or heparin (5 U in 200  $\mu$ l saline) subcutaneously 3 h after LPS administration (3 h before measurement of  $K_p$ ).  $n = 3$  or 4 per group.  $^*P < 0.05$ . **(b)** Pulmonary heparanase expression (red) after CLP in wild-type mice. Images are representative of two or three mice per group. Scale bars, 100  $\mu$ m. **(c)** Mouse pulmonary  $K_p$  measured 48 h after CLP surgery. Wild-type mice received saline (200  $\mu$ l) or heparin (5 U in 200  $\mu$ l saline) subcutaneously 24 h after CLP (24 h before measurement of  $K_p$ ).  $n = 3$  per group.  $^*P < 0.05$ . **(d)** H&E staining of representative lungs of wild-type or  $Hpsr^{-/-}$  mice 48 h after CLP or sham surgery with ensuing exposure to 60% oxygen ( $\text{FiO}_2$ ). Scale bars, 500  $\mu$ m in low-powered images. High-powered imaging (bottom left; scale bar, 50  $\mu$ m) details an area of neutrophilic alveolitis. **(e)** Pulmonary neutrophil infiltration 48 h after CLP and hyperoxia with or without delayed heparin treatment as assessed by histology (top) and myeloperoxidase activity (bottom). Mice were treated with subcutaneous saline (200  $\mu$ l) or heparin (5 U in 200  $\mu$ l saline) 24 h after CLP. Scale bars, 100  $\mu$ m.  $n = 4$  per group.  $^*P < 0.05$ . **(f)** Survival of wild-type ( $n = 14$ ) and  $Hpsr^{-/-}$  ( $n = 6$ ) mice exposed to CLP and 60% hyperoxia. Survival of wild-type mice after sham surgery and 60% hyperoxia was 100% ( $n = 8$ ). Data are represented as means  $\pm$  s.e.m.

and was attenuated by delayed heparin therapy (Fig. 6e). *Hps<sup>-/-</sup>* mice were similarly protected from CLP- and hyperoxia-induced alveolitis (Fig. 6d) and experienced no CLP- and hyperoxia-associated mortality (Fig. 6f).

## DISCUSSION

Our findings demonstrate the contribution of the endothelial glycocalyx to inflammatory diseases such as ALI (Supplementary Fig. 12). In the presence of circulating pathogen-associated molecular patterns, pulmonary microvascular endothelial cells rapidly activate endogenous stores of heparanase. Activated heparanase cleaves heparan sulfate from the pulmonary endothelial glycocalyx, inducing a rapid thinning of the ESL. This ESL loss exposes previously hidden endothelial surface adhesion molecules including ICAM-1 and VCAM-1, allowing neutrophil recognition of and adhesion to the endothelial surface. These events, which pertain to multiple adhesion molecules along the endothelial surface, may work in concert with modifications to individual adhesion molecules (such as increased expression and adhesiveness<sup>25</sup>) to control inflammatory cell influx in the setting of ALI. Consequently, inhibition of heparanase activity represents a potential lung-protective therapy during sepsis.

The glycocalyx has been generally understudied, in part because of the aberrancy of *in vitro* preparations of the ESL and its degradation during tissue handling<sup>26</sup>. Advancements in IVM have coincided with a greater appreciation of glycocalyx and ESL structure and function in health and disease. We examined ESL thickness by dextran exclusion, an IVM technique that allows measurements to be made from images captured at a single timepoint, thereby negating lung motion artifacts. Our findings, to our knowledge representing the first *in vivo* observations of the pulmonary ESL, indicate that pulmonary microvascular ESL thickness (1.67  $\mu\text{m}$ ) is considerably greater than ESL thickness in systemic microvessels (0.67  $\mu\text{m}$ , consistent with previous reports<sup>27</sup>). Our measurements of pulmonary ESL thickness are consistent with prior *in vitro* estimates of glycocalyx thickness (2.8  $\pm$  0.5  $\mu\text{m}$ ) using fluorescence correlation spectroscopy of bovine pulmonary microvascular endothelial cells<sup>28</sup>. The physiological significance of differential ESL size in the systemic and pulmonary microcirculations is uncertain but potentially reflects functional heterogeneity across different vascular beds<sup>29</sup>.

Enzymatic degradation of glycocalyx heparan sulfate rapidly induced a loss of pulmonary ESL thickness, consistent with the dominant role of this GAG in glycocalyx structure. We found that ESL loss similarly occurred during endotoxemia and was mediated by activation of heparanase, an endogenous heparan sulfate-specific glucuronidase. We did not observe concordant endotoxemic ESL loss in the cremaster muscle, a tissue with low levels of heparanase expression (and limited clinical relevance) during sepsis. Extensively studied in cancer metastasis<sup>30</sup>, heparanase has also recently been implicated in inflammatory diseases such as ulcerative colitis<sup>31</sup> and atherosclerosis<sup>32</sup>. Our findings indicate that heparanase also contributes to inflammatory lung injury, with pathologic heparan sulfate degradation arising from post-translational heparanase activation (that is, proenzyme cleavage) as well as a delayed increase in enzyme expression. The proinflammatory consequences of heparanase conflict with a previous report suggesting a protective effect of the 65-kDa heparanase proenzyme as a pretreatment in endotoxemic rats<sup>33</sup>. These seemingly discordant results may be explained by recently described nonenzymatic effects of the heparanase proenzyme<sup>34</sup>. The resistance of *Hps<sup>-/-</sup>* mice (which lack both activated and inactivated heparanase) to septic lung injury suggests that any potential benefits of the endogenous

heparanase proenzyme are outweighed by the untoward enzymatic effects of activated heparanase on the endothelial glycocalyx.

We found that loss of pulmonary ESL thickness exposes endothelial surface adhesion molecules to circulating cells, consistent with a previous study of systemic vessels<sup>10</sup>. Our work reveals key mechanisms underlying ESL regulation of adhesion molecule availability and their impact on inflammatory lung injury. Although the exposure of adhesion molecules is necessary for neutrophil adhesion, additional mechanisms by which glycocalyx loss could induce neutrophil extravasation may also exist. Transgenic mice with constitutively aberrant (desulfated) or truncated heparan sulfate have decreased neutrophil adherence, extravasation or both<sup>35,36</sup>. These findings, combined with our data, suggest that the constitutive absence of heparan sulfate is not sufficient for neutrophil adherence; rather, there must be acute degradation of heparan sulfate from the endothelial glycocalyx. Acute heparan sulfate degradation not only would expose underlying adhesion molecules but also could release proinflammatory mediators (such as the chemokine IL-8) typically sequestered within the ESL, facilitating neutrophil adherence and extravasation<sup>26</sup>.

Pulmonary ESL loss is not an all-or-none phenomenon: a < 0.5- $\mu\text{m}$ -thick glycocalyx remnant remains during endotoxemia. Heparanase may not only decrease ESL thickness but also may increase ESL fluidity, resulting in a more profound effect on barrier function than what is apparent based on measurements of 150-kDa dextran exclusion and perhaps modulating the ability of neutrophils to traverse this remnant ESL. Rigorous testing of this hypothesis will require the development of new *in vivo* techniques to measure ESL stiffness and structural integrity.

We speculate that heparanase-mediated regulation of pulmonary ESL thickness exists to control neutrophil influx into the lung, an organ continuously exposed to airborne pathogens and their associated molecular patterns. In the presence of these danger signals, endothelial cells react by clearing the intimal surface, inviting an influx of inflammatory cells to address the triggering alveolar pathogen. The large size of the pulmonary ESL could reflect the severe consequences of excessive pulmonary neutrophil influx: a thick ESL could dampen an overexuberant response to localized infection, avoiding lung injury and hypoxemia. In sepsis, high concentrations of circulating danger signals might diffusely activate pulmonary ESL degradation, predisposing for inappropriate neutrophilic inflammation and ALI. Inhibition of heparanase activity could therefore represent a new lung-protective intervention in patients with non-pulmonary sepsis. The effect of ESL protection in the setting of a direct injury to the lung (for example, pneumonia) is less certain: heparanase inhibition might oppose the host response to pulmonary infection and be harmful.

If glycocalyx degradation is necessary for the neutrophilic response to danger signals, then glycocalyx reconstitution should also be important during resolution of inflammation. Little, however, is known regarding the mechanisms underlying reconstitution of a damaged glycocalyx. Recovery of cremasteric ESL thickness occurs 72 h after local (high-dose) TNF- $\alpha$  injection<sup>37</sup>; it is unclear whether this recovery period is a function of constitutive GAG turnover (with reconstitution occurring after cessation of the inciting inflammatory insult) or reflects an induction of endothelial repair processes.

In conclusion, our data reveal a crucial role for the pulmonary endothelial glycocalyx in the regulation of neutrophil adhesion to the endothelial surface. In the presence of circulating danger signals, endothelial cells initiate rapid, heparanase-mediated degradation of the ESL, leading to the neutrophilic alveolitis characteristic of ALI.

## METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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## AUTHOR CONTRIBUTIONS

E.P.S. designed all experiments, performed isolated lung experiments, analyzed the data and composed the manuscript. Y.Y. performed IVM, CLP, immunofluorescence and *in vitro* experiments. W.J. analyzed flow cytometry data and circulating neutrophil counts, supervised neutrophil isolation experiments and assisted in manuscript preparation. A.G. performed mouse and human tissue immunofluorescence. M.J.P. and L.P.S. assisted with transgenic mouse breeding and performed lung inflation and fixation. L.B. performed neutrophil isolation experiments, neutrophil flow cytometry and circulating neutrophil counts. R.L.Z. performed neutrophil depletion experiments. J.C.B. performed nuclear factor- $\kappa$ B reporter experiments. D.E.K. performed tissue processing and sectioning. Z.X.Y. performed lung digestion and endothelial flow cytometry experiments. S.S.C. assisted with CLP surgeries. K.H.O., K.R.T. and I.S.D. provided plasma samples from individuals with respiratory failure. M.W.G. obtained support for intravital microscopy. D.B.P. provided support for pilot studies and assisted in manuscript preparation. R.M.T. supervised the overall execution of the project, performed histologic and morphometric analyses and assisted in manuscript preparation.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# Silencing of Irf7 pathways in breast cancer cells promotes bone metastasis through immune escape

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Breast cancer metastasis is a key determinant of long-term patient survival. By comparing the transcriptomes of primary and metastatic tumor cells in a mouse model of spontaneous bone metastasis, we found that a substantial number of genes suppressed in bone metastases are targets of the interferon regulatory factor Irf7. Restoration of Irf7 in tumor cells or administration of interferon led to reduced bone metastases and prolonged survival time. In mice deficient in the interferon (IFN) receptor or in natural killer (NK) and CD8<sup>+</sup> T cell responses, metastasis was accelerated, indicating that Irf7-driven suppression of metastasis was reliant on IFN signaling to host immune cells. We confirmed the clinical relevance of these findings in over 800 patients in which high expression of Irf7-regulated genes in primary tumors was associated with prolonged bone metastasis-free survival. This gene signature may identify patients that could benefit from IFN-based therapies. Thus, we have identified an innate immune pathway intrinsic to breast cancer cells, the suppression of which restricts immunosurveillance to enable metastasis.

Breast cancer can be treated successfully when detected early, but patient survival decreases markedly once metastatic spread to tissues such as bone and lung occurs<sup>1</sup>. Key factors influencing metastases include intrinsic tumor cell factors, the microenvironment, angiogenesis and tumor-specific immune responses<sup>2</sup>. To identify the molecular pathways underlying the interplay between these factors, it is essential to use *in vivo* models. Most studies on the mechanisms of breast cancer metastasis to bone use immunodeficient mouse models, thereby excluding the regulatory activity of the immune system on the metastatic process<sup>3</sup>. There is growing evidence that for outgrowth into clinically detectable tumors, cancer cells must be edited to reduce their immunogenicity and escape the immune recognition that otherwise leads to their elimination or growth inhibition<sup>4-8</sup>. However, there is little evidence on the role of these processes or their regulators in controlling metastasis.

Among the many immunoregulatory cytokines, the type I IFNs are potent regulators of most of the effector cells that are involved in antitumor immune responses<sup>9</sup>. IFNs are produced by immune cells, such as dendritic cells and macrophages, following activation of innate sensors of pathogen infection, including Toll-like receptors (TLRs) and RIG-I-like helicases. These pathways of IFN induction are regulated by IFN regulatory factors (IRF3, IRF5 and IRF7) and nuclear factor of  $\kappa$  light polypeptide gene enhancer in B cells 1 (NF- $\kappa$ B)<sup>9,10</sup>. The biological and pathological effects of IFNs are mediated by signaling through the IFN receptors IFNAR1 and IFNAR2 and the activation of IFN-regulated

genes (IRGs) that encode the effector proteins<sup>11</sup>. To annotate the innate immune pathways that regulate IRG expression changes, we established the INTERFEROME database to predict pathways and transcription factors that regulate specific IRGs or sets of IRGs and their functions, such as inhibition of proliferation or activation of immune effector cells<sup>12</sup>.

We report here our discovery that suppression of an Irf7-driven cluster of innate immune pathway IRGs in breast cancer cells, and not in immune cells, as is typically described, is crucial to the establishment of bone metastases. We used the unique syngeneic 4T1.2 mouse model of spontaneous metastasis from the mammary gland to bone<sup>13,14</sup> to compare the transcriptional profiles of 4T1.2 tumor cells purified from primary mammary tumors and those of matched bone metastases. Interrogation of the list of genes suppressed in metastases using the INTERFEROME database tools<sup>12</sup> revealed a high representation of IRGs, including the transcription factor Irf7 and 208 of its predicted target genes, as well as upstream regulators such as signal transducer and activator of transcription 1 (STAT1). We also show that restored signaling, through enforced Irf7 expression in tumor cells or treatment with type I IFN, enhanced immune activity and significantly suppressed metastasis to bone and prolonged survival time. We found the mechanism of metastasis suppression to involve the modulation of antitumor immune responses, as it was reliant on intact host Ifnar signaling and a competent immune response. Furthermore, analyses of human primary tumors revealed that high

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expression of the *Irf7*-regulated genes in patients with breast cancer was associated with less relapses to bone, thus underscoring the clinical relevance of our findings.

## RESULTS

### Profiling of matched mammary tumors and spine metastases

We isolated tumor epithelial cells from 4T1.2 mouse primary tumors growing in the mammary gland and matched spine metastases<sup>13</sup>, as described previously<sup>15</sup>. We interrogated the differences in gene expression profiles of tumor cells isolated from four matched pairs of primary tumors and bone metastases (Fig. 1) using gene ontology analyses and found them to be highly enriched for immune and defense response ontologies (confidence level  $P < 0.003$ ) (Supplementary Table 1a).

In the spine metastases, 3,547 probes sets representing 3,061 genes were significantly ( $P < 0.05$ ) downregulated and 1,530 probes sets representing 1,012 genes were upregulated at least twofold (Fig. 1). We unambiguously identified genes using Ensembl annotation<sup>16</sup>. An analysis of the 3,061 suppressed genes using the INTERFEROME database identified a high number of IRGs, 540, (Fig. 1 and Supplementary Table 2), which is a significant ( $P < 0.001$ ) overrepresentation. In contrast, there was no significant enrichment of IRGs in the list of upregulated genes. A promoter enrichment analysis of the INTERFEROME gene set (540 genes) identified significant ( $P < 0.001$ ) enrichment of IRF and IFN stimulated gene factor 3 (ISGF3) and of IRF (*Irf7*) ( $P < 0.001$ ), and an additional analysis identified that 208 of the 540 IRGs, including *Irf7*, contained predicted *Irf7* binding sites (Supplementary Figs. 1 and 2).

*Irf7* is a major regulator of the type I IFN pathway<sup>9</sup> and was itself suppressed in tumor cells from bone metastases (Supplementary Table 3). We validated the expression of *Irf7* in primary tumor epithelium, as well as its loss in matched bone metastases, using real-time RT-PCR of epithelial mRNA (Fig. 2a;  $P < 0.001$ ) and immunohistochemistry (Fig. 2b). The marked suppression of both the *Irf7* transcription factor and its 208 putative target genes provided a compelling argument for the functional role of this innate signaling pathway.

The mechanism of loss of *Irf7* expression could stem from direct epigenetic modification (methylation) or a decrease in the expression of components of the ISGF3 complex (which contains STAT1, STAT2 and *Irf9*) that directly regulates *Irf7* expression<sup>17</sup>. Our results indicate the latter option. An analysis of tumor cells macrodissected from 4T1.2 mouse primary tumors and matched spine metastases revealed no methylation at the CpG-rich region spanning exon 1 or at the boundary of intron 1 and exon 2 of *Irf7* in

either type of cells (Supplementary Fig. 3a–c). However, expression of the ISGF3 components STAT1, *Irf9* and STAT2 was lower in bone metastases than in matched primary tumors, correlating with *Irf7* downregulation (Supplementary Table 3). The loss of STAT1 expression was not caused by methylation (Supplementary Fig. 3d). Thus, the mechanism of suppression of *Irf7* expression is probably the downregulation of its upstream regulator: the ISGF3 transcriptional regulator complex. Consistent with this hypothesis, we detected nuclear localization of *Irf9* in primary 4T1.2 tumors (Fig. 2b), which is indicative of ISGF3 translocation to the nucleus. By contrast, nuclear staining was barely detectable in bone metastases, mirroring the results seen with *Irf7* expression (Fig. 2b).

### *Irf7* restores type I IFN signaling and suppresses metastasis

To determine the functional contribution of *Irf7* in bone metastases, we generated 4T1.2 clones overexpressing *Irf7* (4T1.2-*Irf7*; Supplementary Fig. 4a), which did not have altered proliferation *in vitro* (Fig. 3a), even in the presence of pathway stimulation with IFN- $\alpha$  or a TLR3 agonist (poly(I:C)) (data not shown). We confirmed the higher expression of IFN- $\alpha$  in the 4T1.2-*Irf7* clones than in 4T1.2-base vector (BV) control cells that do not overexpress *Irf7* using ELISA (Fig. 3b) and of two IRGs, *Irf9* and STAT1, using quantitative RT-PCR (Supplementary Fig. 4b). The higher expression of the IRGs was the result of secondary signaling by type I IFN, as the administration of an *Irfn1*-neutralizing antibody<sup>18</sup> reduced *Irf9* and STAT1 expression in 4T1.2-*Irf7* cells to the amounts in the 4T1.2-BV control cells (Supplementary Fig. 4b). Indeed, a microarray analysis of gene expression comparing 4T1.2-*Irf7* and 4T1.2-BV cells revealed that all 208 genes that contained predicted *Irf7* binding sites in their promoters were upregulated by *Irf7* expression (Supplementary Fig. 5). Furthermore, there was higher nuclear expression of *Irf9* in 4T1.2-*Irf7* mammary tumors compared to 4T1.2-BV tumors (Supplementary Fig. 4c). Thus, the production of IFN by enforced *Irf7* expression stimulates the IFN-*Irfn1* signal transduction pathway.

We next showed that restoration of the type I IFN pathway by enforced expression of *Irf7* reduced metastatic burden in the mouse

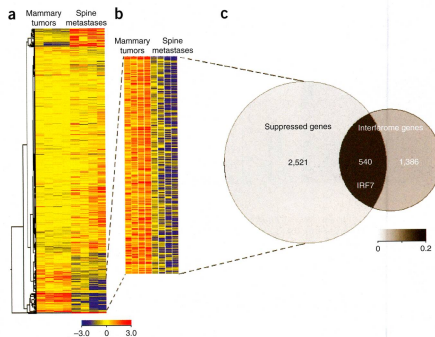
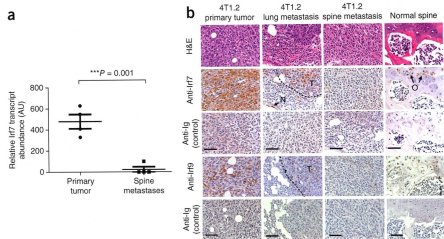


**Figure 1** Bone metastases derived from breast carcinomas downregulate interferon pathways and other immune-related genes. Shown are heatmaps generated using hierarchical clustering and applying Euclidean distance and the centroid linkage rule. The colored scale bar represents the  $\log_2$ -transformed fold change. The gray bar represents the CLOVER enrichment score (Online Methods). *Irf7* was identified as a regulatory gene in the intersection of the suppressed genes and the INTERFEROME genes.



**Figure 2** Expression of the transcription factor *Irf7* is suppressed in bone metastases. **(a)** Real-time RT-PCR analysis for *Irf7* expression in mRNA isolated from 4T1.2 primary tumors and spine metastases (error bars, s.e.m.;  $n = 4$  samples per group). The *Irf7* transcript abundance is relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).  $P = 0.001$  by *t* test. AU, arbitrary units. **(b)** Immunohistochemical staining of tissue sections taken from BALB/c mice bearing 4T1.2 tumors (primary tumors, lung metastases and spine metastases) and control mice (normal spine). Serial sections were stained with H&E for morphology (top), *Irf7*-specific antibodies (anti-*Irf7*), preimmune rabbit Ig control, *Irf9*-specific antibodies (anti-*Irf9*) or rat IgG control antibodies. T, tumor; N, normal lung epithelium; O, osteocytes (as indicated by arrows). Scale bars, 50  $\mu$ m. The dashed lines indicate the boundaries of the tumors.



model. Enforced expression of *Irf7* did not affect the growth rates of the primary tumors (Fig. 3c). Notably, overexpression of *Irf7* resulted in a significant reduction in metastasis to spine (Fig. 3d), as measured by PCR and visualized by histology. We detected spine macrometastases in 60% of mice bearing 4T1.2-BV tumors, yet these macrometastases were undetectable in mice bearing 4T1.2-*Irf7* tumors (Fig. 3e).

To confirm that the *Irf7*-mediated reduction in metastasis can prolong survival and is dependent on type I IFN signaling to host cells, we inoculated 4T1.2-BV or 4T1.2-*Irf7* cells into wild-type (WT) or *Irfar1*<sup>-/-</sup> BALB/c<sup>19</sup> mice. Although there was no difference in primary tumor growth between any of the groups (Fig. 3f), increased expression of *Irf7* resulting from inoculation of 4T1.2-*Irf7* cells led to a significantly ( $P = 0.03$ ) prolonged metastasis-free survival time

in the WT mice (Fig. 3g). In contrast, *Irfar1*<sup>-/-</sup> mice succumbed to metastasis faster in both inoculation groups, and enforced *Irf7* expression did not suppress metastasis (Fig. 3g). Crucially, bone metastases were evident in all *Irfar1*<sup>-/-</sup> mice (Fig. 3h).

Taken together, these results show that the IFN signaling in tumor cells that is driven by *Irf7* reduces metastasis to bone and that the host response through *Irfar1* is crucial for the suppression of metastasis and the associated prolonged survival time.

### *Irf7* expression restores antimetastatic immune responses

Because the host IFN response is crucial for metastasis suppression and the innate IFN pathway regulates the development and activity of immune effector cells<sup>5</sup>, we assessed their potential roles in the 4T1.2

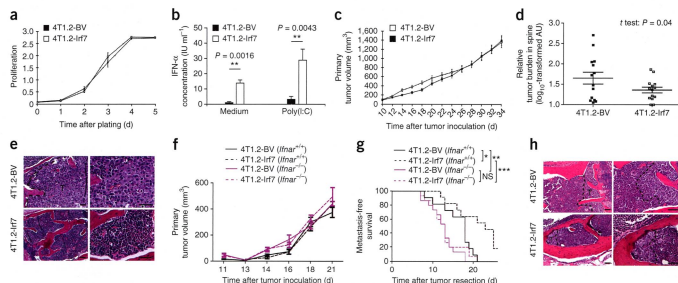


**Figure 3** Restoration of *Irf7* expression enhances IFN signaling and inhibits metastasis. **(a)** *In vitro* proliferation assay of 4T1.2-BV and 4T1.2-*Irf7* cells.  $n = 6$  per group. Error bars, s.e.m. **(b)** ELISA for IFN- $\alpha$  of conditioned medium from 4T1.2-BV or 4T1.2-*Irf7* cells with and without stimulation with poly(I:C).  $n = 6$  cells per group. Error bars, s.e.m. **(c)** Tumor volumes after inoculation of 4T1.2-BV or 4T1.2-*Irf7* cells into the mammary glands of syngeneic, immunocompetent BALB/c mice. Error bars, s.e.m. **(d)** Quantitative real-time PCR detection of metastatic tumor burden in spines of mice bearing 4T1.2-BV or 4T1.2-*Irf7* tumors (with the endpoint of the experiment at day 32). **(e)** H&E-stained sections of spines from immunocompetent BALB/c mice bearing 4T1.2-BV or 4T1.2-*Irf7* tumors. Scale bars, 50  $\mu$ m. T, tumor. The dashed line indicates the boundaries of the tumors. **(f)** Tumor volumes after inoculation of 4T1.2-BV and 4T1.2-*Irf7* tumor cells into litter-matched *Irfar1*<sup>-/-</sup> and WT mice. Error bars, s.e.m. **(g)** Metastasis-free survival in WT and *Irfar1*<sup>-/-</sup> mice harboring 4T1.2-BV or 4T1.2-*Irf7* tumors.  $n = 12$  (4T1.2-BV *Irfar1*<sup>-/-</sup>),  $n = 13$  (4T1.2-*Irf7* *Irfar1*<sup>-/-</sup>),  $n = 16$  (4T1.2-BV *Irfar1*<sup>+/+</sup>) or  $n = 16$  (4T1.2-*Irf7* *Irfar1*<sup>+/+</sup>).  $^{*}P = 0.0123$ ,  $^{**}P = 0.0067$ ,  $^{***}P = 0.0002$ , NS, not significant by log-rank test. **(h)** H&E-stained sections of spines from *Irfar1*<sup>-/-</sup> mice bearing 4T1.2-BV or 4T1.2-*Irf7* tumors at the experiment end point. Scale bars, 50  $\mu$ m. T, tumor. The dashed lines indicate the boundaries of the tumors.

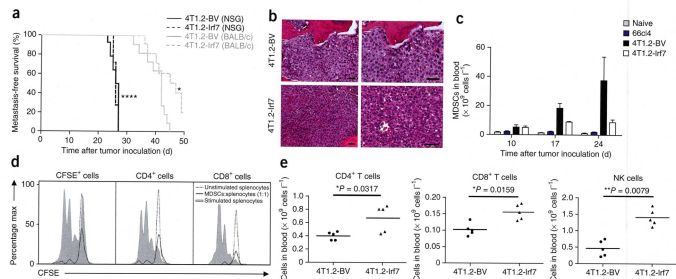


**Figure 4** Modulation of the immune system by metastatic tumor cells and reversion of these effects by enforced expression of the type I IFN pathway. (a) Disease-free survival of mice bearing 4T1.2-BV or 4T1.2-Irf7 tumors on either the immunocompetent BALB/c background (the data from Fig. 3g are reproduced in light gray for comparison) or the immunocompromised NSG background. \*\*\*\*, not significant, \* $P = 0.0123$  by log-rank test.  $n = 15$  mice per group. (b) H&E-stained sections of spines from NSG mice bearing 4T1.2-BV or 4T1.2-Irf7 tumors at the endpoint of the experiment. Scale bars, 50  $\mu\text{m}$ ; the images on the right are magnifications of the images on the left. (c) Accumulation of MDSCs over time in the peripheral blood of BALB/c mice bearing no tumor (naive) or 66c4, 4T1.2-BV or 4T1.2-Irf7 tumors ( $n = 3$  mice per group). Data are means  $\pm$  s.e.m. (d) Carboxyfluorescein succinimidyl ester (CFSE)-labeled naive BALB/c splenocytes were activated with antibodies to CD3 and CD28 and cocultured with or without CD11b<sup>+</sup>Ly6G<sup>+</sup> MDSCs (ratio 1:1) derived from the mice bearing 4T1.2-BV tumors. Total splenocytes, CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation at 72 h was measured by flow cytometry. A two-tailed Student  $t$ -test was performed, and the mean fluorescence intensity for CFSE was compared between the splenocytes  $\pm$  MDSC of seven replicate samples. (e) The numbers of CD4<sup>+</sup> T cells (CD4<sup>+</sup>TCR- $\beta$ <sup>+</sup>), CD8<sup>+</sup> T cells (CD8<sup>+</sup>TCR- $\beta$ <sup>+</sup>) and NK cells (DX5<sup>+</sup>TCR- $\beta$ <sup>+</sup>) in the peripheral blood of mice inoculated with 4T1.2-BV or 4T1.2-Irf7 tumors 5 d earlier. (f) Tumor volumes of mice bearing 4T1.2-Irf7 tumors depleted of CD8 cells, NK cells or both. (g) Disease-free survival rates of mice depleted of CD8 cells, NK cells or both. No depletion ( $n = 14$ ), CD8 depletion ( $n = 11$ ), NK-cell depletion ( $n = 7$ ), NK- and CD8-cell depletion ( $n = 11$ ). \* $P = 0.0114$ , \*\* $P = 0.0014$  (for no depletion compared to NK- + CD8-cell depletion), \*\* $P = 0.0047$  (for NK-cell depletion compared to NK- + CD8-cell depletion), NS, not significant by log-rank test.

model by inoculating 4T1.2-BV or 4T1.2-Irf7 cells into nonobese diabetic severe combined immunodeficient (NOD-SCID) interleukin 2 receptor  $\gamma$  (IL-2 $\gamma$ )-null mice (NSG) and WT BALB/c mice. The primary tumors grew at the same rate in both NSG and WT groups, suggesting that primary tumor growth is not restrained by the immune system. Notably, NSG mice lacking mature lymphocytes succumbed to metastasis more rapidly than WT BALB/c mice, and disease-free survival time was not extended by enforced expression of Irf7 (Fig. 4a); these results contrast with the outcome found in immunocompetent BALB/c mice, in which Irf7 conferred a significant ( $P = 0.03$ ) survival benefit (Fig. 4a). These data show that functional immune cells are necessary for Irf7 to confer protection against metastasis. A histological examination revealed spine metastases in all NSG mice (Fig. 4b), which is consistent with their reduced survival times. These results show that metastatic tumors are restrained by the immune system and that Irf7 possibly suppresses metastasis by restoring antitumor immune responses without affecting primary tumor growth.

To determine the immune cells mediating the Irf7-regulated modulation of bone metastases, we first examined systemic parameters of immune function. Mice bearing 4T1.2-BV tumors had a marked elevation of peripheral white blood cells and splenomegaly compared to

naive mice. This response was not as strong in mice bearing 4T1.2-Irf7 tumors (Supplementary Fig. 6). Previous studies in the 4T1 model have detected a high number of myeloid-derived suppressor cells (MDSCs) compared to naive mice, which suppress immune surveillance to promote tumor growth<sup>20,21</sup>. We measured Gr1<sup>+</sup>CD11b<sup>+</sup> MDSCs in the peripheral blood of both groups of tumor-bearing mice and detected an increased population of these cells in mice bearing the highly metastatic 4T1.2-BV tumors relative to those bearing the 4T1.2-Irf7 tumors or the nonbone metastatic variant, 66c4 (Fig. 4c). We also visualized the infiltration of MDSCs into 4T1.2-Irf7 and 4T1.2-BV tumors using immunohistochemical staining for Gr1-positive cells (Supplementary Fig. 7a). We detected a greater number of Gr1<sup>+</sup> cells in the bone metastases from mice bearing 4T1.2-BV tumors than in the primary tumors and lung metastases from these mice (Supplementary Fig. 7b). We did not detect any bone metastases in mice bearing 4T1.2-Irf7 tumors, and, therefore, we could not assess the effect of enforced Irf7 expression on MDSC infiltration of bone metastases. As MDSCs consist of two major subsets, lymphocyte antigen 6 complex, locus G (Ly6G)<sup>+</sup>CD11b<sup>+</sup> neutrophilic MDSCs and Ly6G<sup>+</sup>CD11b<sup>+</sup> monocytic MDSCs<sup>22</sup>, we measured both cell populations, which revealed a markedly higher number of

Ly6G<sup>hi</sup> neutrophilic MDSCs in all mice bearing 4T1.2 tumors than in naive mice. The number of Ly6G<sup>hi</sup>CD11b<sup>+</sup> cells was lower in mice with 4T1.2-Irf7 tumors compared to those with 4T1.2-BV tumors (Supplementary Fig. 8). To show the activity of Ly6G<sup>hi</sup>CD11b<sup>+</sup> MDSCs, we isolated these cells from the peripheral blood of mice bearing 4T1.2 tumors and found that they significantly inhibited the proliferation of CD4<sup>+</sup> ( $P = 0.0021$ ) and CD8<sup>+</sup> ( $P = 0.0021$ ) lymphocytes (Fig. 4d). These data show that the MDSC population that is lower in mice bearing 4T1.2-Irf7 tumors than in those bearing 4T1.2-BV tumor is functionally capable of suppressing lymphocyte activity. Previous reports linked tumor MDSCs with granulocyte colony stimulating factor (G-CSF)<sup>23,24</sup>. We found significantly ( $P < 0.05$ ) lower primary tumor expression and plasma concentrations of G-CSF in mice bearing 4T1.2-Irf7 tumors than in those bearing 4T1.2-BV or nonbone metastatic 66c4 tumors (Supplementary Fig. 9).

The lower number of MDSCs in mice bearing 4T1.2-Irf7 tumors (and the associated decrease in the amount of G-CSF), along with the ability of type I IFNs to directly enhance T cell and NK cell effector functions<sup>25</sup>, suggests that enhanced tumor cell recognition may be involved in the suppression of metastasis that is mediated by Irf7. Consistent with this idea, we found significantly more CD4<sup>+</sup>, CD8<sup>+</sup> and NK cell populations in the peripheral blood of mice bearing 4T1.2-Irf7 tumors compared to those bearing 4T1.2-BV tumors (Fig. 4e) 5 d after tumor cell inoculation, when tumor cells have probably intravasated into the circulation but have not yet formed metastases. To test their function, we depleted CD8<sup>+</sup> T cells and NK cells from mice bearing either 4T1.2-Irf7 or 4T1.2-BV tumors. Consistent with all previous *in vivo* experiments, primary tumor growth was unaltered by the depletion of either or both cell types (Fig. 4f). However, depletion of both CD8<sup>+</sup> T cells and NK cells significantly ( $P = 0.0014$ ) accelerated metastasis and shortened survival time in mice with 4T1.2-Irf7 tumors (Fig. 4g), indicating that both of these effector cells contribute to the Irf7- and IFN-mediated control of metastases. Notably, we found a clear increase in the incidence of paralysis in these mice caused by the development of bone metastases, which we confirmed by histology (Supplementary Fig. 10). These data confirm that the Irf7-induced and IFN-dependent immune suppression of metastasis to bone was dependent on CD8<sup>+</sup> T cells and NK cells.

### IRF7 pathway in human breast cancer

We next sought to determine whether IRF7 was expressed in human primary breast cancers and suppressed in bone metastases.

Using immunohistochemistry, we verified the expression of IRF7 in human primary tumors and its loss in distant metastases, including bone, using tissue arrays derived from the Rapid Autopsy Program at Johns Hopkins Hospital<sup>26</sup>. We found that IRF7 was frequently expressed in normal breast tissue (7/10) and in primary tumors (9/16). Notably,

we found lower IRF7 expression in the metastases, with only 16.7% of distant metastases expressing IRF7 (3/18) ( $P < 0.04$  relative to primary tumors; Supplementary Table 4). An analysis of matched primary tumors and bone metastases within this cohort revealed that in the nine pairs, seven primary tumors expressed IRF7, only one of which also had expression in the matched bone metastasis (Fig. 5a,b). Similar to what we found in mouse tissues (Fig. 2b), bone osteocytes in these human cancers expressed IRF7, whereas the tumor cells did not, in most cases.

To determine the clinical relevance of the IRF7 pathway in human breast cancers, we used publicly available gene expression datasets of 855 primary tumors with a known location of the first site of distant metastases<sup>27,28</sup>. Using the 208 genes previously predicted as being regulated by Irf7 (Fig. 1 and Supplementary Fig. 1), we evaluated associations between this Irf7 signature and relapses to bone and nonbone as the first site of distant metastases. Notably, we found that the Irf7 signature was significantly (adjusted hazard ratio of 0.63 (95% CI 0.42–0.93),  $P = 0.021$ ) associated with bone-metastases-free survival in a linear manner, with low expression of the Irf7 gene set being associated with a higher number of bone metastatic events (Fig. 5c); we found no such relationship for other sites, including the brain, lung and liver (Fig. 5d). The ability to predict bone metastases was independent of estrogen receptor status, lymph node involvement, tumor size and histological grade. Irf7 expression alone was not a predictor of bone metastasis (data not shown). Overall, these results support the mouse data that the Irf7 pathway is crucial for the suppression of bone metastases in human breast cancer.

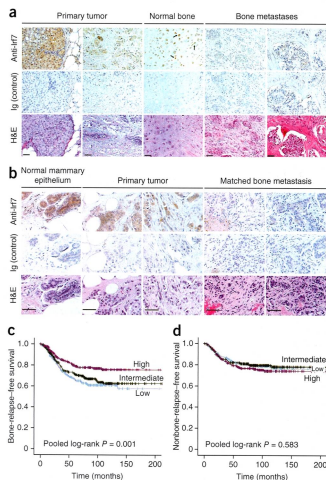
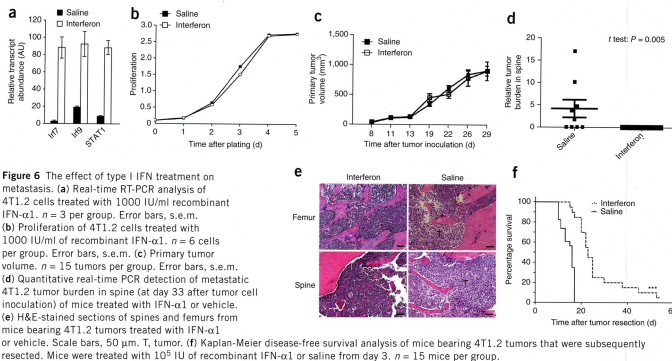


**Figure 5** IRF7 expression in human breast cancer tissues. (a) Samples of unmatched primary breast tumors and bone metastases, as well as normal bone stained for IRF7, Ig control and H&E (as indicated). Arrows indicate osteocyte staining. Scale bars, 50  $\mu$ m. (b) Primary breast tumor and a matched bone metastasis stained for IRF7, Ig control and H&E. Also shown is normal mammary epithelium from a breast reduction mammoplasty for comparison. Scale bars, 50  $\mu$ m. (c) IRF7 pathway activity as measured by gene expression in human breast cancers and significant association with bone metastases as the first site of distant relapse. Purple, black and blue indicate the highest, intermediate and lowest tertile of expression of the Irf7 gene signature, respectively. The log-rank  $P$  value indicated was calculated using data pooled across the three strata. (d) The Irf7 gene signature is not significantly associated with nonbone relapses (brain, liver or lung).



### Treatment with IFN- $\alpha$ 1 suppresses metastasis

As metastasis suppression by Irf7 was reliant on type I IFN, we sought to determine whether IFN administration would reverse the metastatic burden in bone. Treatment with IFN- $\alpha$ 1 increased the expression of Irf7, as well as of Irf9 and STAT1, in 4T1.2 cells *in vitro* (Fig. 6a), which is consistent with our data that showed that Irf7 increased Irf9 and STAT1 expression through IFN. As we expected from our data described above, treatment with IFN- $\alpha$ 1 did not affect 4T1.2 cell proliferation *in vitro* (Fig. 6b) or primary tumor growth *in vivo* (Fig. 6c). In stark contrast, IFN treatment significantly reduced metastasis to the spine and femur to undetectable amounts ( $P < 0.007$ ) (Fig. 6d,e). The extent of metastasis to the lung was not reduced by treatment with IFN (Supplementary Fig. 11). We found less accumulation of MDSCs and lower concentrations of G-CSF in the peripheral blood of IFN-treated mice compared to untreated mice (Supplementary Fig. 12). For the metastasis-free survival studies, we treated mice bearing 4T1.2 tumors with either saline or IFN- $\alpha$ 1 for 5 weeks and performed primary tumor resection at 3 weeks. IFN- $\alpha$ 1 had no effect on the growth of primary tumors but resulted in a significant ( $P < 0.0001$ ) extension of metastasis-free survival time (Fig. 6f). At individual end points, we assessed the mice for metastatic burden and all succumbed to bone and lung metastases. These data show that therapeutic administration of IFN- $\alpha$ 1 can reduce metastatic burden and is a viable therapeutic strategy that is worthy of further investigation.

To provide insights into the mechanism of IFN inhibition of bone metastases specifically, we measured the effect of IFN treatment on immune cell populations in 4T1.2-tumor-bearing mice. The percentage of CD11b<sup>+</sup>Ly6G<sup>+</sup> MDSCs in the bone marrow was far greater than that in the lungs and mammary glands of these mice (Supplementary Fig. 13). IFN treatment significantly decreased the number of MDSCs in the bone marrow ( $P = 0.0079$ ) and peripheral blood ( $P = 0.0119$ ) (Supplementary Figs. 12 and 13a) but not in the mammary glands and primary tumors or in the lungs of the mice (Supplementary Fig. 13). Additionally, the number of NK cells was significantly ( $P = 0.0159$ ) increased in the bone marrow of the mice

after IFN treatment, an effect that we did not find at the primary tumor site or in the lungs of the tumor-bearing mice (Supplementary Fig. 13a). These data show that IFN reduces the number of immune suppressor cells and increases the number of effectors specifically in the bone marrow. In addition, NK cells from the mammary glands and lungs of BALB/c mice were predominantly CD11b<sup>high</sup>CD27<sup>low</sup> (Supplementary Fig. 13b), which is indicative of terminally differentiated cells that have a high threshold for stimulation<sup>29</sup>. In contrast, the majority of NK cells from the bone marrow of mice were CD11b<sup>low</sup>CD27<sup>high</sup> (Supplementary Fig. 13b), representing NK cells with greater effector function that are reportedly more sensitive to stimulation by cytokines such as IFN.

Together these data imply that the mechanism whereby IFN specifically inhibits bone metastases of mammary cancers is by selective modulation of MDSCs and NK effectors in bone marrow.

### DISCUSSION

The unique aspect of this study is the discovery of an innate immune pathway in tumor cells that is fundamental for the outcome of metastasis. Our bioinformatic analyses using the INTERFEROME database revealed that 540 IRGs were suppressed in bone metastases, including 208 that have predicted binding sites for the master regulator of type I IFN responses, Irf7 (ref. 9) which is autoregulated. Restoration of this signature suppressed the spread of mammary tumors to bone and was dependent on a competent immune response. Because activation of this IRF7 pathway is usually associated with innate immune responses to infection in immune cells, the activation of this pathway in tumor cells was noteworthy. Of the downstream effectors, type I IFN was crucial in metastasis suppression, as *Irfn1* deficiency in the host accelerated metastasis and prevented the suppressive effects of Irf7.

IFNs are well-characterized modulators of the development and function of most effector cells of the immune system (reviewed in refs. 30–32), and thus, can regulate tumor immunosurveillance and elimination<sup>32,33</sup>. For example, recent studies in a transgenic prostate adenocarcinoma model showed that activation of Tlr3 signaling

increased NK cell activity and suppressed prostate tumor growth through an IFNAR-dependent mechanism<sup>34</sup>. Type I IFNs and some IRFs have also been implicated in direct tumor suppression through the induction of apoptosis and antiproliferative responses<sup>30,35</sup>. Accordingly, IFN- $\beta$  is used therapeutically in the treatment of a number of cancers, including metastatic melanoma and metastatic renal carcinoma<sup>36,37</sup>. However, it should be noted that most of these findings address primary tumors, and there is limited prior evidence for any role of the type I IFN pathway in metastases of breast or other cancers, which is the key finding of our study. A recent study has implicated another Irf expressed in tumor cells, Irf5, in breast cancer invasion<sup>38</sup>. However, it should be noted that in that study, the role of type I IFN in the inhibition of tumor cell growth and invasion was not evaluated, and the observed inhibitory effects were intrinsic to tumor cells and independent of immune regulatory mechanisms. In our model of bone metastasis, Irf5 was not altered, and it was not regulated by Irf7 expression (data not shown).

The mechanism of metastasis suppression by Irf7 expression in tumor cells is caused by activation of tumor immune surveillance mechanisms, as the effect of Irf7 was completely abolished in immunocompromised NSG mice. Notably, mice bearing 4T1.2-Irf7 tumors (or other non-metastatic tumors) had a reduction in the number of MDSCs, which we previously linked with the type I IFN pathway<sup>19</sup>. Recent evidence indicates that MDSCs are crucial for cancer immune escape and are potent suppressors of T and NK cell function<sup>39–41</sup>. Increased numbers of MDSCs have been observed in a number of metastatic mouse mammary carcinoma models, including the MMTV-PyMT<sup>42</sup>, MMTV-Her2/neu<sup>43</sup> and 4T1 (refs. 20,21,44) models; a functional role for promotion of metastasis by MDSCs was shown in the 4T1 model. Thus, in our study, Irf7-induced IFN production might prevent the accumulation of MDSCs, allowing the suppression of metastases by NK cells and T lymphocytes. Not only did we detect elevated concentrations of NK and CD8<sup>+</sup> T cells in the peripheral blood of tumor-bearing mice, but depletion of these tumor immune surveillance<sup>8</sup> cell populations abolished the metastasis-suppressive effects of Irf7.

Our data are consistent with clinical studies reporting that transplant patients treated with immunosuppressive therapies do not have an increased incidence of breast cancer<sup>45,46</sup>, suggesting that tumor immunosurveillance does not regulate the initiation of primary breast tumors. This hypothesis is also supported by other groups using breast cancer models, where effects on metastases were independent of primary tumor initiation and growth<sup>47,48</sup>. In support of a role of immune suppression in promoting metastasis, accumulation of circulating MDSCs in human cancer correlates with disease progression and metastasis<sup>41,49</sup>, including breast cancer, where detection of MDSCs correlated with clinical cancer stage and metastatic tumor burden<sup>50</sup>. Our data further suggest that low expression of IRF7 signatures in the primary breast tumor may highlight those patients at high risk for the development of bone metastases, supporting other reports of the expression of metastasis-regulating genes in the primary tumor<sup>27,51,52</sup>. These results are also timely given that bone modulating therapies are currently being evaluated in the adjuvant treatment of breast cancer for the primary prevention of bone metastases<sup>53,54</sup>. The means to identify high-risk patients may facilitate better selection of the individuals who would benefit from such therapies.

IFN- $\alpha$ 1 significantly reduced metastasis to bone and prolonged metastasis-free survival in our mouse model. This survival advantage prompts a discussion about the potential use of type I IFNs or IRF7/TLR agonists as therapeutics to prevent metastatic outgrowth in breast cancer. Interestingly, a subset of human primary tumors was recently

reported to express an IRG cluster<sup>55</sup>, consistent with our data that IRF7 is expressed in primary breast cancers (where it would drive IRG expression). Future studies will be required to determine the value of IRF7 and the IRF7 signature in predicting patients that may benefit from IFN-based therapies. There are limited reports of type I IFN treatment in patients with breast cancer that were restricted to small cohorts or to terminally ill patients who had extensive metastases. In these cohorts, IFN treatment did not prolong survival<sup>56,57</sup>. This may be because the timing of treatment was late, when macrometastases had already developed. We hypothesize that the relevant tumor immunosurveillance checkpoint that can be activated by type I IFN is the rate-limiting step in metastasis during the transition from disseminated cells to outgrowth into overt metastases<sup>50</sup>. This hypothesis needs to be tested in the future to evaluate the utility of IFN-based therapies in breast cancer.

We report for the first time, to our knowledge, that breast carcinoma cells have intrinsic Irf7-driven innate immune pathways, suppression of which enables escape from immunosurveillance and the formation of overt metastases in bone. We show that suppression of metastasis can be achieved by restoring innate IFN signaling in tumor cells and the activation of innate and acquired immune responses. Our data will prompt future research into the therapeutic potential of antimetastatic immunotherapies targeting IFN pathways in patients with breast cancer and dissection of the molecular markers in primary tumors that may determine which patients are most likely to respond to such therapies and benefit from prophylactic treatment.

## METHODS

Methods and any associated references are available in the online version of the paper.

**Accession codes.** Microarray data from the comparison of 4T1.2-BV and 4T1.2-Irf7 cells (**Supplementary Fig. 5**) have been deposited in Gene Expression Omnibus under the accession code GSE37828, and microarray data indicated in **Figure 1** are deposited under accession code GSE37975.

*Note: Supplementary information is available in the online version of the paper.*

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## AUTHOR CONTRIBUTIONS

B.N.B. and C.Y.S. generated the majority of data in the manuscript. N.P.W. and A.M. contributed to mouse experiments, including intramammary fatpad injections and mouse tissue harvests. Y.C., D.A. and N.E.M. carried out the fluorescence-activated cell sorting (FACS) analysis on immune populations. T.M. did the methylation-sensitive high-resolution melting (MS-HRM), S.E. (Ph.D. student and graduate of computer science and genetics graduate, Monash University) and S.A.S. (bioinformatician) carried out the bioinformatics analysis of the mouse microarray experiments and promoter analyses. S.L., a clinician scientist with expertise in bioinformatics and biostatistics, carried out the prognostic analyses. P.A. is a pathologist who provided and scored the matched primary tumor and metastases tissue arrays. N.A.D.W. produced, purified and tested IFN- $\alpha$ 1 for *in vivo* experiments. J.G. performed some microarray experiments.



P.J.H. and B.S.P. were responsible for project design, interpretation of data and drafting the manuscript, in collaboration with R.L.A. and M.J.S.

# COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# Neural precursor cells induce cell death of high-grade astrocytomas through stimulation of TRPV1

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**Primary astrocytomas of grade 3 or 4 according to the classification system of the World Health Organization (high-grade astrocytomas or HGAs) are preponderant among adults and are almost invariably fatal despite the use of multimodal therapy. Here we show that the juvenile brain has an endogenous defense mechanism against HGAs. Neural precursor cells (NPCs) migrate to HGAs, reduce glioma expansion and prolong survival time by releasing endovanilloids that activate the vanilloid receptor (transient receptor potential vanilloid subfamily member-1 or TRPV1) on HGA cells. TRPV1 is highly expressed in tumor and weakly expressed in tumor-free brain. TRPV1 stimulation triggers tumor cell death through the branch of the endoplasmic reticulum stress pathway that is controlled by activating transcription factor-3 (ATF3). The antitumorigenic response of NPCs is lost with aging. NPC-mediated tumor suppression can be mimicked in the adult brain by systemic administration of the synthetic vanilloid arvanil, suggesting that TRPV1 agonists have potential as new HGA therapeutics.**

Somatic mutant neural stem cells and NPCs are thought to be the source of HGAs, one of the most aggressive forms of tumors of the central nervous system (CNS)<sup>1</sup>. A very large fraction of individuals diagnosed with HGAs and glioblastomas (GBMs) are adults<sup>2–3</sup>. However, adult neurogenesis (that is, the presence and activity of NPCs in the postnatal and adult brain) is maintained at a high rate only during infancy and declines thereafter<sup>4,5</sup>. Hence, the epidemiology of GBMs and the timing of adult neurogenesis are inversely correlated, and GBMs are often diagnosed several decades after the decline in brain stem cell activity.

It has been previously found that endogenous and exogenous NPCs have a strong tropism for primary brain tumors and that NPCs can release tumor-suppressive factors<sup>6–13</sup>. However, the molecular nature of the factors that mediate cell death in HGA cells has not been elucidated. We show that NPCs induce tumor cell death through the release of endovanilloids. Endovanilloids<sup>14</sup>, such as arachidonoyl ethanolamide (AEA) and *N*-arachidonoyl dopamine (NADA), directly stimulate the vanilloid receptor, TRPV1, which is abundantly expressed on HGA<sup>15</sup>. TRPV1 is a nonselective cation channel that is best characterized in capsaicin-sensitive sensory neurons of the dorsal root and trigeminal ganglia<sup>16</sup>.

Synergistic TRPV1 activation by AEA in combination with other fatty acid ethanolamides, such as oleoyl ethanolamide (OEA) or palmitoyl ethanolamide (PEA), has previously been reported<sup>15,16</sup>. The physiological role of the ion channel in non-neural tissues is largely unexplored<sup>16</sup>. Here we show that endovanilloid release from NPCs activates TRPV1 on HGA cells and thereby induces tumor-cell death.

## RESULTS

### NPCs induce HGA cell death through TRPV1

We investigated the signaling pathways that are activated in HGA cells after exposure to NPC-conditioned medium (NPC-CM). An analysis of gene expression changes (by microarrays of stimulated and control HGA cells; GSE37671) together with pharmacological studies using specific TRPV1 antagonists and molecular studies using specific TRPV1 knockdown in HGA cells suggested a role for NPC-derived endovanilloids as tumor suppressors. Furthermore, we observed by real time PCR that TRPV1 expression is positively correlated with grading in human primary brain tumors and that TRPV1 expression in tumors was higher than in human tumor-free brain (Supplementary Fig. 1a).

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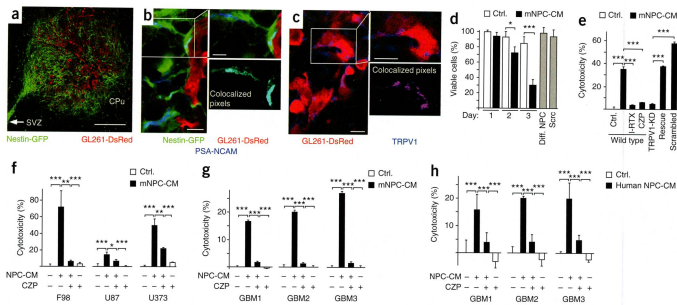


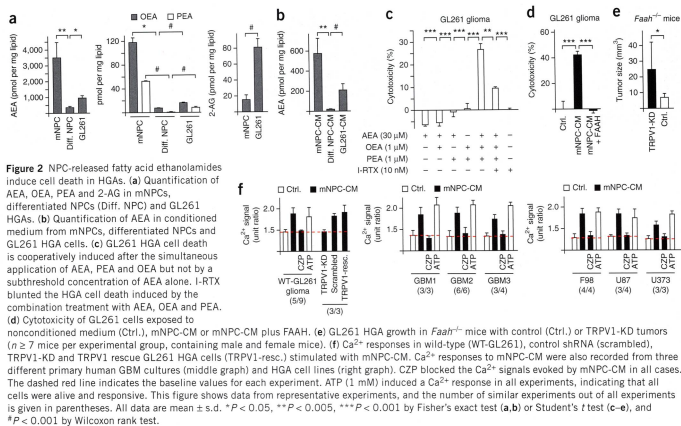
**Figure 1** TRPV1 agonists released from NPCs induce HGA cell death. **(a)** Large numbers of nestin-GFP<sup>+</sup> cells are present after 14 d of tumor development at a DsRed<sup>+</sup> glioma in the caudate putamen (CPu) of young mice (tumor implantation on postnatal day 30;  $n = 12$ , containing male and female mice for each immunohistochemistry). The arrow points to the subventricular zone (SVZ). Scale bar, 500  $\mu$ m. **(b)** Glioma-associated nestin-GFP<sup>+</sup> cells express PSA-NCAM (blue). A single cell (within the boxed area in the image on the left) is magnified on the right, and colocalizing pixels of a single optical section are shown. Scale bar, left image, 10  $\mu$ m; magnified image, 6  $\mu$ m. **(c)** Glioma cells are immunopositive for TRPV1 (blue). A single cell (within the boxed area in the image on the left) is magnified on the right, and colocalizing pixels of a single optical section are shown. Scale bars, 10  $\mu$ m. **(d)** The viability of mouse GL261 glioma cells is reduced after stimulation with mNPC-CM but not after stimulation with nonconditioned medium (Ctrl.) or other control media (gray bars). Diff, NPC, differentiated NPCs; srcr, nontumorigenic fibroblasts. **(e)** The mNPC-CM-induced cytotoxicity of GL261 cells was blocked by CZP and the knockdown of TRPV1 (TRPV1-KD) but not by a control shRNA (scrambled); rescue of the TRPV1-KD fully restored the effect of mNPC-CM. **(f–h)** The relative cytotoxicity of primary human GBM cells **(g,h)** and HGA cell lines **(f)** after incubation with mNPC-CM **(f,g)** or human NPC-CM **(h)** with or without CZP. All data are mean  $\pm$  s.d. \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.001$  by Student's  $t$  test.

We next explored the role of the endovanilloid system in NPC-mediated HGA suppression in an established mouse model<sup>8,9,17</sup>. We orthotopically implanted brain tumor cells (DsRed-expressing GL261 cells) in nestin-GFP mice, which are a model for the visualization of NPCs<sup>8,18</sup> (Fig. 1a). Fourteen days after tumor cell injection, we found that subventricular NPCs migrated to HGAs that were located in the caudate putamen in these mice<sup>8,9,17</sup>. We identified NPCs using colocalization of GFP with established immunocytochemical markers, such as polysialylated neuronal cell adhesion molecule (PSA-NCAM) or Musashi<sup>19,20</sup>. PSA-NCAM is also a marker of tumor-associated NPCs in humans (S.M., unpublished observations). Notably, we found that the mouse HGAs had higher amounts of immunolabeling for TRPV1 (Fig. 1c) than tumor-free brain, whereas only a small fraction of tumor-associated nestin-GFP<sup>+</sup> cells expressed TRPV1, and TRPV1 was absent from subventricular NPCs in the mice (Supplementary Fig. 1b).

In a series of *in vitro* experiments, we found that stimulation of mouse HGAs with factors released from mouse NPCs (mNPC-CM), but not with factors released from their fully differentiated progeny (astrocytes, oligodendrocytes and neurons) or from fibroblasts (srcr), strongly reduced the viability of HGA cells over a time-course of 3 d (Fig. 1d; the data for the conditioned medium from the control cells were obtained on day 3). In subsequent experiments, we stimulated the mouse HGA cells for 3 d, unless indicated otherwise. Stimulation with mNPC-CM reduced the viability of the HGA cells by inducing cell death, as indicated by TUNEL and cytotoxicity assays (Supplementary Fig. 1c). All cytotoxicity values are expressed as a percentage of the fully permeabilized cells (Supplementary Figs. 2 and 3).

HGAs in the GL261 mouse glioma model express TRPV1 and contain specific binding sites for a selective TRPV1 ligand (Supplementary Fig. 1d,e). In this model, HGA cell death induced by stimulation with mNPC-CM was greatly reduced by blocking TRPV1 with the selective antagonists<sup>16</sup> idoforesiniferatoxin (I-RTX, 10 nM; Fig. 1e) or capsaizine (CZP, 1  $\mu$ M; Fig. 1e) and by knockdown of TRPV1 (TRPV1-KD; Fig. 1e). For the knockdown of TRPV1, we expressed five different TRPV1-directed shRNAs and control shRNA (scrambled) in GL261 cells and then selected for vectors that block TRPV1 function in GL261 cells. We subsequently quantified TRPV1 protein expression by quantitative proteomics (selected reaction monitoring, SRM) in knockdown and control GL261 cells (Supplementary Fig. 1d). SRM allowed for a direct, specific and well-controlled quantification of TRPV1 protein expression. We verified the efficiency and specificity of the TRPV1 knockdown (Supplementary Fig. 1d,e) by performing experiments with control shRNAs (scrambled shRNA) and with overexpression of a knockdown-resistant form of mouse TRPV1 (ref. 21) in TRPV1-KD HGA cells (Fig. 1e,f and Supplementary Fig. 2c). Exposure to mNPC-CM strongly induced cell death in various HGA cell lines (Fig. 1f) and in three different primary human GBM cultures (Fig. 1g), an effect that was blocked by the simultaneous application of either I-RTX (Supplementary Figs. 2b and 3a,b) or CZP. Notably, human NPC-CM also induced cell death in primary human GBMs after stimulation of TRPV1 (Fig. 1h).

Overall, our cell-culture experiments showed that human and mouse NPCs release TRPV1 agonists, which induce HGA cell death. Our data from a mouse model showed that, in the young brain, many NPCs accumulate at HGAs. An analysis of TRPV1 expression in



HGAs and tumor-free brain from mice and humans indicated that HGAs have high expression of TRPV1, whereas we found low TRPV1 expression in non-neoplastic CNS.

### NPCs constitutively release endovanilloids

We quantified the concentrations of AEA, NADA, OEA, PEA and the endocannabinoid arachidonoyl glycerol (2-AG) in samples from mNPCs, fully differentiated progeny from mNPCs and mouse HGA cells. Differentiation of NPCs was achieved by growth factor withdrawal and transient addition of fetal bovine serum. After differentiation, we maintained the mNPC progeny in stem cell medium (as used for nondifferentiated mNPCs) and collected the conditioned media. The cell densities in the differentiated and undifferentiated NPCs were equivalent. Using mass-spectrometry, we found that mNPCs contained markedly high amounts of AEA, PEA and OEA, whereas the amounts of these endovanilloids in the differentiated mNPCs and the mouse HGA cells were much lower (Fig. 2a). NADA was not detectable in any of the samples (data not shown). We found high concentrations of AEA in the mNPC-CM (Fig. 2b), whereas the culture supernatants from the differentiated mNPCs or the HGA cells contained much less AEA, and the concentrations of other lipids were below the detection limit. We observed that the combined application of synthetic AEA, PEA and OEA exerts a cooperative effect on HGA cell death<sup>22</sup>, which was blocked by the addition of I-RTX (Fig. 2c). The addition of fatty acid amide hydrolase (FAAH), which degrades ethanolamides<sup>23</sup>, fully abolished the effect of mNPC-CM (Fig. 2d) and of human NPC-CM to induce cell death (data not shown).

We detected only low concentrations of the endocannabinoid 2-AG in mNPCs (Fig. 2a; notably, 2-AG concentrations were higher in HGAs than in NPCs). Stimulation of HGAs with the vanilloid

NADA induced cell death, and exposure of HGAs to mNPC-CM containing specific cannabinoid receptor antagonists did not blunt the HGA cell death (Supplementary Figs. 2f,g and 3c,d). Together, these data suggest a role for endovanilloids (rather than endocannabinoids) as mediators of NPC-induced HGA cell death<sup>22</sup>. Next, we used FAAH-deficient (*Faah*<sup>-/-</sup>) mice, which have much higher amounts of endocannabinoids and endovanilloids in the CNS than wild-type mice<sup>24</sup>, as a brain tumor model. *Faah*<sup>-/-</sup> mice are an ideal model to elevate endogenous levels of fatty acid amides. However, the experiments with *Faah*<sup>-/-</sup> mice foreclosed any comparison to wild-type mice, because, for example, neural stem and immune cells have different activity in *Faah*<sup>-/-</sup> mice than wild-type mice. Thus, we investigated the effect of TRPV1 signaling on glioma growth by implanting TRPV1-KD or control HGAs into *Faah*<sup>-/-</sup> mice. We found that implantation of TRPV1-KD tumors resulted in much larger tumors than did implantation of control HGAs in these mice (Fig. 2e). These data support our finding from the *in vitro* experiments and show that even greatly elevated concentrations of endogenous endocannabinoids and endovanilloids exert their tumor-suppressive effects exclusively through TRPV1 receptors in our glioma model.

TRPV1 is abundantly expressed in dorsal root ganglion neurons (DRGs)<sup>25</sup>, and opening of the TRPV1 channel induces a cation influx, which can be measured, for example, as a rise in the intracellular concentration of free calcium ions. To assess the activity of endovanilloids released from NPCs, we designed a bioassay using DRGs from wild-type and *Trpv1*<sup>-/-</sup> mice<sup>26</sup>. We stimulated the DRGs from both groups with mNPC-CM and measured the responses using Fura-2-based calcium imaging. We observed that 5.4% of the wild-type DRGs responded to both an initial stimulation with NPC-CM and to a subsequent stimulation with the selective TRPV1 agonist

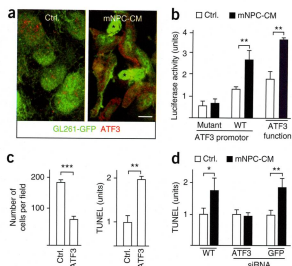


**Figure 3** TRPV1 agonists released by NPCs trigger the ATF3 pathway in HGAs. (a) ATF3 expression in HGAs (GL261 cells expressing GFP, GL261-GFP) exposed to nonconditioned medium (Ctrl.) or mNPC-CM; notably, the cell morphology changed after exposure to NPC-CM, and the small and rounded GL261 cells (asterisk) seem damaged. Scale bar, 10  $\mu$ m. (b) A wild-type form of the ATF3 gene promoter (WT) was activated in GL261 cells after stimulation with NPC-CM, whereas a mutant control of the ATF3 gene promoter (mutant) was not. A gene promoter with an ATF3 binding site (ATF3 function) is also induced after stimulation with mNPC-CM. (c) Overexpression of ATF3 reduced GL261 cell density and induced DNA fragmentation (determined by TUNEL) as compared to GL261 cells expressing empty vector (control). (d) siRNA for ATF3 prevents mNPC-CM-induced nuclear strand breaks in GL261 HGA cells. All data are mean  $\pm$  s.d. \* $P$  < 0.05, \*\* $P$  < 0.005, \*\*\* $P$  < 0.001 by Student's  $t$  test.

capsaicin. Only 0.85% of wild-type DRGs responded to initial stimulation with NPC-CM alone but not to the subsequent stimulation with capsaicin. In *Trpv1*<sup>-/-</sup> mice, only 0.91% of the DRGs responded to mNPC-CM, which would suggest that TRPV1 is required for the majority of the responses to mNPC-CM observed in the wild-type DRG neurons (Supplementary Fig. 4a). In addition, RT-PCR analysis of cultured mNPCs, their differentiated progeny and whole-brain extracts revealed that mNPCs express the major receptors and metabolic enzymes of the endovanilloid and endocannabinoid pathways (Supplementary Fig. 4b). These data support the view that endovanilloids are major constituents of mNPC-CM and that endovanilloids are released from NPCs in physiologically relevant concentrations.

Furthermore, mNPC-CM stimulated TRPV1-mediated Ca<sup>2+</sup> responses in primary human GBM cultures and in human, rat and mouse HGA cell lines (Fig. 2f and Supplementary Fig. 4c). By performing pharmacological experiments and immunoelectron microscopy, we observed that TRPV1 in the GL261 HGAs was ectopically expressed in the endoplasmic reticulum (ER; Supplementary Fig. 5b–e). It has been previously described that TRPV1 in the ER has

a lower sensitivity for capsaicin than TRPV1 located in the plasma membrane<sup>16,27</sup>. We also found a lower sensitivity to capsaicin in our experiments (Supplementary Fig. 4c).

### TRPV1 induces cell death through ER stress

We next investigated the gene expression pattern in mouse tumor cells after incubation with nonconditioned medium (controls) or with mNPC-CM by microarrays (GSE37671). We found that ER stress genes, such as the activating transcription factor-3 gene (*ATF3*), were robustly upregulated in mouse HGA cells treated with mNPC-CM compared to controls. Immunocytochemical labeling and reporter gene assays in GL261 cells treated with mNPC-CM showed that the expression of ATF3 was higher in both the cytoplasm and nucleus of these cells than in controls and that mNPC-CM activates an ATF3-responsive element in a gene promoter (Fig. 3a,b). Forced expression of ATF3 reduced the number of GL261 cells in culture and increased the number of TUNEL<sup>+</sup> tumor cells (Fig. 3c). Notably, siRNA-mediated downregulation of ATF3 expression (Supplementary Fig. 6a) in mouse HGA cells prevented the tumor cell death induced by mNPC-CM (Fig. 3d). Therefore, ATF3 is necessary and sufficient for mediating the HGA cell death induced by mNPC-CM. Administering the TRPV1 antagonist CZP blocked the mNPC-CM-induced activation of the ATF3-dependent ER stress pathway in mouse HGAs (Supplementary Fig. 6b,c).

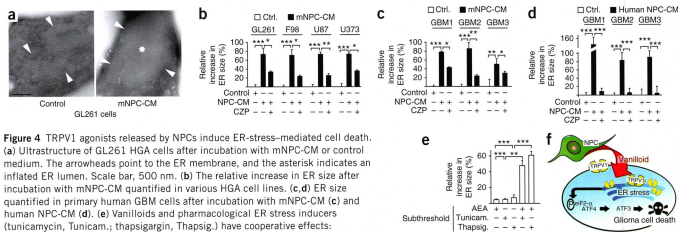


**Figure 4** TRPV1 agonists released by NPCs induce ER-stress-mediated cell death. (a) Ultrastructure of GL261 HGA cells after incubation with mNPC-CM or control medium. The arrowheads point to the ER membrane, and the asterisk indicates an inflated ER lumen. Scale bar, 500 nm. (b) The relative increase in ER size after incubation with mNPC-CM quantified in various HGA cell lines. (c) ER size quantified in primary human GBM cells after incubation with mNPC-CM (c) and human NPC-CM (d). (e) Vanilloids and pharmacological ER stress inducers (tunicamycin, Tunicam., thapsigargin, Thapsig.) have cooperative effects: subthreshold concentrations of the combined substances induce ER enlargement. (f) Illustration of NPCs constitutively releasing endovanilloids (fatty acid ethanolamides such as AEA, PEA and OEA), which traverse the plasma membrane of HGAs and stimulate TRPV1 by docking to an intracellular receptor binding site. NPC-induced TRPV1 activation (mainly located in the ER; Supplementary Fig. 5) triggers the ATF3-dependent ER stress pathway in HGAs, which includes activation (phosphorylation, indicated by the P within a circle) of eukaryotic initiation factor 2  $\alpha$  (eIF2- $\alpha$ ) and ATF4 (ref. 28; Supplementary Fig. 6). Increased expression of ATF3 is necessary and sufficient to mediate HGA cell death. All data are mean  $\pm$  s.d. \* $P$  < 0.05, \*\* $P$  < 0.005, \*\*\* $P$  < 0.001 by Student's  $t$  test.



Electron microscopy revealed that GL261 cells treated with mNPC-CM had an enlarged ER as compared to controls (Fig. 4a and Supplementary Fig. 6d), which is a morphological hallmark of ER stress<sup>28</sup>. We quantified the effect of mNPC-CM (with or without the addition of CZP) on the ER in primary human GBM and in human, rat and mouse HGA cell lines using ER-tracker (Fig. 4b,c). We then determined the effect of human NPC-CM on ER size in primary human GBMs (Fig. 4d). In all HGA cells studied, we detected a very robust increase in ER size after stimulation with human or mouse NPC-CM, which was always attenuated by the addition of CZP (Fig. 4b–d and Supplementary Fig. 7). Additionally, we used synthetic AEA and inducers of ER stress, such as tunicamycin or thapsigargin<sup>29</sup>, at concentrations that were below the threshold for ER stress induction when applied alone. We found that the simultaneous application of AEA and either tunicamycin or thapsigargin led to strong increases in ER size in GL261 cells (Fig. 4e). The combined substances had a clear cooperative effect on the rise in ER size, confirming that vanilloid-induced signaling and ER stress are part of the same signal transduction pathway in HGA cells. These data show that human and mouse NPC-derived endovanilloids induce HGA cell death through the ER stress pathway (Fig. 4f).

### Age dependency of NPC-induced tumor suppression

To investigate whether NPC-derived endovanilloids can suppress HGAs *in vivo*, we performed orthotopic implantation of HGAs into nestin-GFP mice of different ages. Implantation of GL261 cells (TRPV1-KD or controls, which express a nontargeting shRNA) into young (30-day-old) mice resulted in the association of many

endogenous NPCs to the tumor<sup>8,9,17</sup> (Fig. 5a). Nestin-GFP<sup>+</sup> NPCs accumulated at GL261 control HGAs and TRPV1-KD HGAs in equal densities. Notably, we found that young mice injected with TRPV1-KD cells had significantly larger (70%) tumors compared to young mice injected with GL261 control cells (Fig. 5b). We found no difference in tumor size in 90-day-old adult mice administered GL261 control cells and those administered TRPV1-KD cells (Fig. 5b). In another set of experiments, we used a transgenic mouse model to manipulate the numbers of endogenous NPCs independently of aging. Therefore, we used cyclin D2 knockout (*Ccnd2*<sup>-/-</sup>) mice, which have substantially lower amounts of adult neurogenesis than do wild-type mice<sup>9,30</sup>. In these *Ccnd2*<sup>-/-</sup> mice and their wild-type littermates, we orthotopically implanted GL261 cells (control or TRPV1-KD) and measured the tumor size 14 d after implantation. We found that the tumor size in the wild-type mice receiving control HGAs was at least 63% smaller than the tumors in the wild-type mice receiving TRPV1-KD tumor cells or *Ccnd2*<sup>-/-</sup> mice receiving either control HGAs or TRPV1-KD tumor cells (Fig. 5c). We then measured HGA cell death *in vivo* by systemically delivering propidium iodide (Fig. 5d)<sup>31</sup>. We noted that TRPV1-KD HGAs had much lower levels of HGA cell death than control HGAs.

In another set of experiments, we tested the effect of endovanilloids released from NPCs on the overall survival of a cohort of wild-type mice with HGAs. First, we orthotopically implanted control or TRPV1-KD GL261 cells into young mice and compared the cumulative survival times of the two groups. We observed that young wild-type tumor-bearing mice (Fig. 5e) had a significantly longer survival time after implantation than the older mice. However, when we

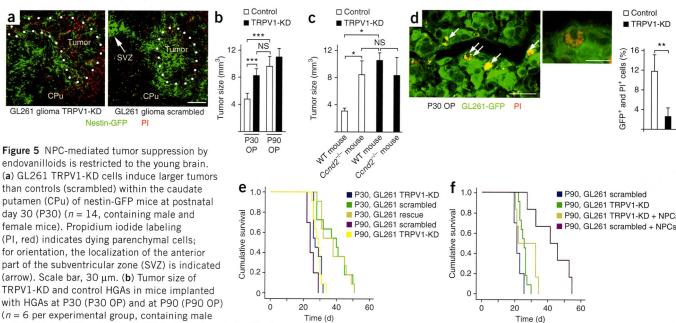


**Figure 5** NPC-mediated tumor suppression by endovanilloids is restricted to the young brain. (a) GL261 TRPV1-KD cells induce larger tumors than controls (CPU) of nestin-GFP mice at postnatal day 30 (P30) ( $n = 14$ , containing male and female mice). Propidium iodide labeling (PI, red) indicates dying parenchymal cells; for orientation, the localization of the anterior part of the subventricular zone (SVZ) is indicated (arrow). Scale bar, 30  $\mu\text{m}$ . (b) Tumor size of TRPV1-KD and control HGAs in mice implanted with HGAs at P30 (P30 OP) and at P90 (P90 OP) ( $n = 6$  per experimental group, containing male and female). (c) Tumor size of TRPV1-KD and control HGAs in wild-type or *Ccnd2*<sup>-/-</sup> mice implanted with HGAs at P30; *Ccnd2*<sup>-/-</sup> mice have reduced neurogenesis ( $n = 4$  mice per experimental group, containing male and female). (d) Nuclear propidium iodide labeling indicates dying GL261-GFP cells (arrows), and a single PI<sup>+</sup> tumor cell is magnified (double arrows) in the image on the right. Quantification of propidium iodide-labeled (dying) tumor cells. Implantation of TRPV1-KD tumors led to less HGA cell death than implantation of wild-type tumors ( $n = 14$  mice, male and female). Scale bar, left, 30  $\mu\text{m}$ ; right, 10  $\mu\text{m}$ . (e, f) The proportion of P30 (or P90) surviving HGAs, that is, inoculation with control (scrambled or rescue) or TRPV1-KD GL261 cells. Mice implanted at P30 outlive mice implanted at P90, unless the mice implanted at P30 were given TRPV1-KD tumor cells ( $n \geq 10$  mice per group, male and female; e). Implantation of P90 mice with both NPCs and control HGAs (scrambled) improves survival but co-injection of NPCs with TRPV1-KD HGAs does not ( $n \geq 6$  mice per group, male and female; f). All data are mean  $\pm$  s.d. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , NS, not significant by Student's  $t$  test. In e and f, survival was statistically significantly different, with  $P < 0.001$ : in e, this comparison was between the 'P30, GL261-scrambled' or 'P30, GL261-rescue' group and all other groups, and in f, this comparison was between the 'P30, GL261-scrambled + NPCs' group and all other groups.

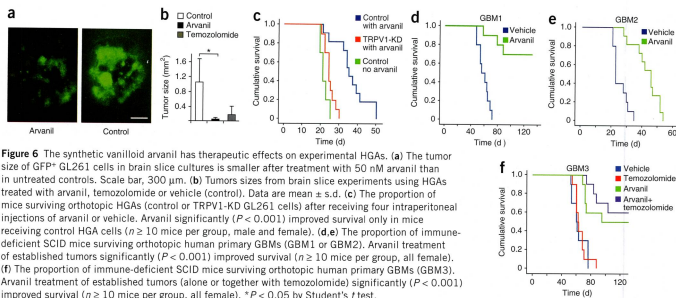


**Figure 6** The synthetic vanilloid arvanil has therapeutic effects on experimental HGAs. **(a)** The tumor size of GFP<sup>+</sup> GL261 cells in brain slice cultures is smaller after treatment with 50 nM arvanil than in untreated controls. Scale bar, 300  $\mu$ m. **(b)** Tumor sizes from brain slice experiments using HGAs treated with arvanil, temozolomide or vehicle (control). Data are mean  $\pm$  s.d. **(c)** The proportion of mice surviving orthotopic HGAs (control or TRPV1-KD GL261 cells) after receiving four intraperitoneal injections of arvanil or vehicle. Arvanil significantly ( $P < 0.001$ ) improved survival only in mice receiving control HGA cells ( $n \geq 10$  mice per group, male and female). **(d,e)** The proportion of immune-deficient SCID mice surviving orthotopic human primary GBMs (GBM1 or GBM2). Arvanil treatment of established tumors significantly ( $P < 0.001$ ) improved survival ( $n \geq 10$  mice per group, all female). **(f)** The proportion of immune-deficient SCID mice surviving orthotopic human primary GBMs (GBM3). Arvanil treatment of established tumors (alone or together with temozolomide) significantly ( $P < 0.001$ ) improved survival ( $n \geq 10$  mice per group, all female). \* $P < 0.05$  by Student's *t* test.

implanted young mice with TRPV1-KD HGA cells they survived, on average, the same amount of time as older mice. These data show that younger mice have an intrinsic protective mechanism against HGAs that is dependent on endovanilloid signaling. In a second study, we investigated whether the survival-promoting effect that we observed could be attributed specifically to NPCs. Therefore, we implanted adult mice with exogenously cultivated NPCs and control HGAs or TRPV1-KD tumor cells (Fig. 5f). We found that implantation of control HGAs always generated tumors but that co-implantation of NPCs together with HGAs prolonged survival (Fig. 5e,f;  $P < 0.001$ ). We also found that the survival-promoting effect of NPCs in adult mice was absent after implantation with TRPV1-KD cells (Fig. 5f).

Our data suggest that NPCs release endovanilloids *in vivo* in a way similar to NPCs *in vitro*. Consistently, the extent of the NPC-mediated antitumor response depended on the amount of adult neurogenesis.

### Synthetic vanilloids as therapeutics for HGAs

We next investigated the therapeutic potential of the synthetic, non-pungent, blood-brain-barrier-permeable vanilloid arvanil<sup>32,33</sup>. We allowed control HGAs to develop for 5 d in organotypic brain slice cultures obtained from wild-type mice. The addition of temozolomide<sup>24</sup> (200  $\mu$ M, which is the current standard of care for the treatment of patients with GBM) or arvanil (50 nM) strongly reduced the size of the HGAs as compared to vehicle-treated controls (Fig. 6a,b). Furthermore, arvanil induced a TRPV1-dependent  $\text{Ca}^{2+}$  signal and TRPV1-dependent cell death in the HGAs (Supplementary Fig. 8). In further experiments, we orthotopically implanted TRPV1-KD or control HGAs into wild-type mice and treated both groups with arvanil as described above. We compared the survival of these arvanil-treated mice to a cohort of mice receiving control HGA cells and no arvanil treatment. We found that the mice receiving the control HGAs plus arvanil had significantly longer survival time than the mice receiving TRPV1-KD tumor cells plus arvanil (Fig. 6c;  $P < 0.001$ ) or control tumors without arvanil. These data indicate that arvanil elicits its therapeutic effect as a TRPV1 agonist. To determine whether arvanil would also lead to longer survival in other HGA models, we implanted primary human GBM cells (GBM1 and GBM2) into severe combined immunodeficient (SCID) mice. At 1 week after implantation, we examined the tumor development in the mice and administered arvanil

(a total of four intraperitoneal injections of 1 mg kg<sup>-1</sup> body weight each) or vehicle. Treatment with arvanil substantially prolonged survival as compared to treatment with vehicle (Fig. 6d–f;  $P < 0.001$ ). We then compared the effects of the application of arvanil and temozolomide (alone and in combination) on survival after implantation of a third primary human HGA culture (GBM3) in immune deficient (SCID) mice. We found that arvanil prolonged survival in a cohort of SCID mice that received GBM3 cells that did not respond to temozolomide (given once daily for 5 d at 100 mg kg<sup>-1</sup> body weight (ref. 35); Fig. 6f;  $P < 0.001$ ). These data show the potential clinical value of an experimental HGA therapy using vanilloids, which may also offer a new therapeutic option for temozolomide-resistant HGAs<sup>36</sup>.

### DISCUSSION

We have shown that HGAs have high expression of TRPV1 and that TRPV1 stimulation induces tumor cell death. Neural stem and precursor cells home in on HGAs and release antitumorigenic TRPV1 agonists (endovanilloids). Endogenous and exogenous NPCs show extensive tropism for brain tumors<sup>6–10</sup>. However, the number of endogenous NPCs accumulating at HGAs depends on the proliferative activity in the stem cell niche and declines before the onset of adulthood<sup>9</sup>. Hence, the recruitment of large numbers of NPCs to a tumor, and the concomitant antitumorigenic release of endovanilloids, is mostly restricted to the young brain. Additionally, other age-related changes in neural stem cell physiology may also impinge on the ability of NPCs to suppress tumors<sup>37,38</sup>.

We show here that NPCs are a primary source of endogenous TRPV1 and cannabinoid receptor agonists such as AEA<sup>15,39</sup>. This finding was supported by our detection of high amounts of AEA and related acylethanolamides in undifferentiated NPCs, as well as our findings that factors released by NPCs evoke TRPV1-dependent  $\text{Ca}^{2+}$  responses in DRGs and HGAs, that the tumor-suppressive effect of NPC-CM is lost after the addition of FAAH and that NPC-induced HGA cell death is dependent on TRPV1 *in vitro* and *in vivo*. These data are in agreement with previous reports indicating that synthetic AEA induces HGA cell death<sup>40</sup>.

A role for TRP channels in tumor suppression was previously suggested by us and others<sup>41–45</sup>, but the present study is the first, to our knowledge, to identify NPCs as a cellular source for

tumor-suppressive endovanilloids and uncover the role of TRPV1 agonists and modulators released by NPCs on HGA cell death. Overall, our study suggests that endovanilloids are intrinsic tumor suppressors in the brain and that synthetic vanilloid compounds may have clinical potential for the treatment of brain tumors.

## METHODS

Methods and any associated references are available in the online version of the paper.

**Accession codes.** Microarray data are deposited in Gene Expression Omnibus under the accession code GSE37671.

*Note: Supplementary information is available in the online version of the paper.*

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## AUTHOR CONTRIBUTIONS

S.P.R., A.L., L.D.P., U.G. and S.R.C. designed and conducted the experiments, and interpreted the data. K.S., J.K., E.S.I., P.W., B.P., U.A.N., V.M., B.F.C., S.M., V.D.M., J.-H.W., G.D. and L.C. contributed to manuscript preparation. G.R.L., V.D.M. and H.K. designed the experiments, supervised the project, interpreted the data and contributed to manuscript preparation. M.S. performed brain tumor resections and provided tumor samples. M.S. and R.G. designed and conducted the experiments, supervised the project, interpreted the data and prepared the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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## MDM4 is a key therapeutic target in cutaneous melanoma

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The inactivation of the p53 tumor suppressor pathway, which often occurs through mutations in *TP53* (encoding tumor protein 53) is a common step in human cancer. However, in melanoma—a highly chemotherapy-resistant disease—*TP53* mutations are rare, raising the possibility that this cancer uses alternative ways to overcome p53-mediated tumor suppression. Here we show that Mdm4 p53 binding protein homolog (MDM4), a negative regulator of p53, is upregulated in a substantial proportion (~65%) of stage I–IV human melanomas and that melanocyte-specific Mdm4 overexpression enhanced tumorigenesis in a mouse model of melanoma induced by the oncogene *Nras*. MDM4 promotes the survival of human metastatic melanoma by antagonizing p53 proapoptotic function. Notably, inhibition of the MDM4–p53 interaction restored p53 function in melanoma cells, resulting in increased sensitivity to cytotoxic chemotherapy and to inhibitors of the *BRAF* (V600E) oncogene. Our results identify MDM4 as a key determinant of impaired p53 function in human melanoma and designate MDM4 as a promising target for antimelanoma combination therapy.

Cutaneous melanoma is the leading cause of skin cancer–related deaths and is notorious for its resistance to therapy. Nevertheless, recent targeted therapy trials have been promising<sup>1,2</sup>. Notably, the Ras/Raf/MEK/ERK pathway has been identified as a major, druggable regulator of melanoma<sup>3</sup>. Activating *NRAS* mutations are commonly observed in human melanoma, usually affecting codon 61 (refs. 4,5). *BRAF* is also frequently mutated<sup>6</sup>, most commonly resulting in a glutamic acid for valine substitution at position 600 (V600E) (ref. 6).

*BRAF* (V600E), which results in constitutively overactive MAPK/ERK signaling and melanocyte hyperproliferation<sup>7</sup>, has been successfully exploited for targeted therapy. PLX4032 (vemurafenib), a selective RAF inhibitor, showed an unprecedented antitumor response rate in patients with *BRAF* (V600E) (ref. 8) and conferred an overall survival benefit in a pivotal phase 3 study<sup>9</sup>. Unfortunately, most patients rapidly acquire resistance to vemurafenib<sup>10</sup>, highlighting the urgent need for new treatment strategies of *BRAF* (V600E)-induced melanoma.

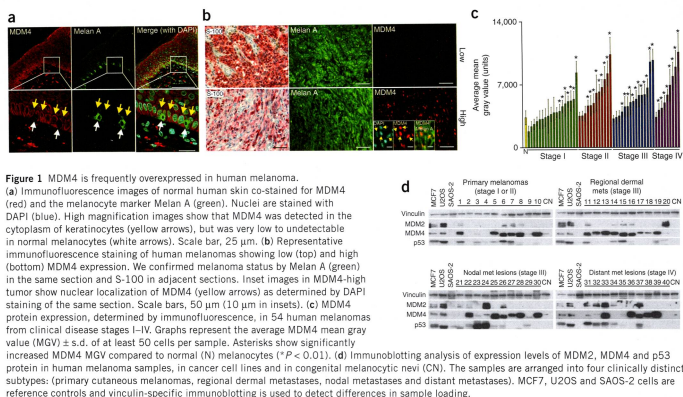
Restoration of the wild-type p53 tumor suppressor function has emerged as an attractive anticancer strategy<sup>11–13</sup>. However, usefulness

of this approach in melanoma is unclear; although inactivating mutations or allelic loss of *TP53* are common in human cancers<sup>14</sup>, the *TP53* locus is intact in >95% of melanomas<sup>15</sup>. Nevertheless, increasing evidence supports a role for p53 in melanomagenesis, as loss of p53 cooperates with activated HRAS<sup>V12G</sup> and *BRAF*<sup>V600E</sup> in mice<sup>16,17</sup> and with oncogenic *NRAS* in zebrafish<sup>18</sup>, culminating in melanoma formation.

Cancers that retain wild-type p53 often find alternative ways to subvert p53 function, by deregulating upstream modulators and/or by inactivating downstream effectors<sup>19</sup>. For example, MDM2, which encodes an E3 ubiquitin ligase that controls p53 expression and function<sup>20</sup>, is amplified in 3–5% of human melanomas<sup>21</sup>.

Here we show that the p53 pathway is inactivated in the majority of cutaneous melanomas as a result of upregulated expression of MDM4, a key negative regulator of p53 (refs. 22,23). We show that targeting the MDM4–p53 interaction inhibits the growth of melanoma cells and markedly sensitizes them to conventional chemotherapeutics. Notably, MDM4 inhibition affects the growth of melanoma cells that have acquired resistance to *BRAF* inhibitors and synergizes with *BRAF*

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inhibitors to kill BRAF-mutant cells. These data identify the MDM4-p53 interaction as a new therapeutic target for melanoma treatment.

## RESULTS

### MDM4 is overexpressed in ~65% of human melanomas

To investigate whether MDM4 overexpression contributes to p53 inactivation in human melanomas, we assessed MDM4 protein expression in normal skin and in melanomas by immunofluorescence. Although detectable in the cytoplasm of epidermal keratinocytes, MDM4 expression was very low to undetectable in normal melanocytes (Fig. 1a). Assessment of a panel of 54 primary cutaneous and metastatic melanomas showed variable MDM4 expression (from very low or undetectable to high), with MDM4 localizing to the nucleus in most cells (Fig. 1b). We observed that MDM4 expression was significantly increased in 68.5% of melanomas compared with normal melanocytes (Fig. 1c), including 56.7% of early stage (stage I or II) tumors and 83.3% of metastatic (stage III or IV) tumors (Fig. 1c). We found no correlation between age (calculated as age at resection of tumor) or pathologic subtype and MDM4 expression (Supplementary Table 1). There was, however, a statistically significant correlation between high MDM4 expression and late-stage (stage III or IV) metastatic disease (Supplementary Table 1).

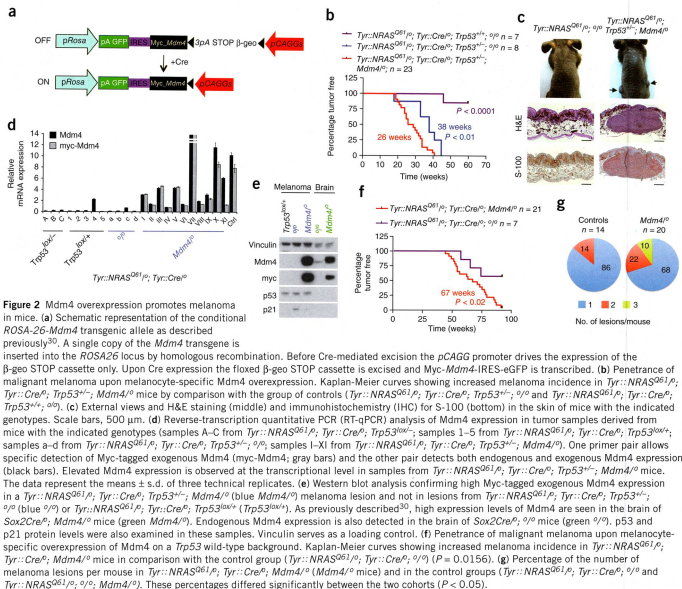
We confirmed MDM4 overexpression in an additional cohort of 40 freshly isolated human melanomas by immunoblotting (Fig. 1d). Notably, in only 3 out of 40 of these samples were MDM4 mRNA levels comparable to or higher than those in the breast cancer cell line MCF-7, which is known to express high MDM4 mRNA levels (Supplementary Fig. 1)<sup>24</sup>. In contrast, MDM4 protein expression was comparable to or higher than that observed in MCF-7 cells in 65% of cases (Fig. 1d and Supplementary Table 2). Consistent with the immunofluorescence data, MDM4 protein expression was either undetectable or very low in normal melanocytes and in benign nevi

(Fig. 1d and Supplementary Fig. 2a). Six out of ten primary cutaneous tumors had high MDM4 levels (Fig. 1d), supporting the possibility that MDM4 upregulation occurs early in melanomagenesis. In contrast, MDM2 protein expression levels ranged from undetectable to low in most cases (Fig. 1d). We only found MDM2 expression levels comparable to those in U2OS cells, an osteosarcoma cell line highly expressing MDM2, in one out of ten regional dermal metastases, one out of ten nodal metastases and four out of ten distant metastases (Fig. 1d and Supplementary Table 1). Overexpression of MDM2 and MDM4 co-occurred in only 2 out of 30 metastatic melanomas (stage IV) (Fig. 1d).

Compared with primary melanocytes, we found that MDM4 was also elevated in 14 out of 16 patient-derived short-term cultures established from metastatic tumors, as well as in four out of four cell lines (A375, WM9, Mel-501, Lu1205) harboring wild-type p53 (Supplementary Fig. 2b,c and Supplementary Table 3). Consistent with the notion of a post-transcriptional mechanism being primarily responsible for MDM4 upregulation, MDM4 mRNA levels were higher than those in MCF-7 in only one of these cell lines (MM120; Supplementary Fig. 1b). As in freshly isolated human melanoma samples, MDM2 protein expression levels ranged from undetectable to low in the majority of short-term cultures (Supplementary Fig. 2b,c). We found that MDM2 levels were comparable to those in U2OS cells in four cell lines (MM011, MM034, MM061, MM117) and higher in only two cell lines (MM001, MM120; Supplementary Table 3). Notably, MDM2 was highly expressed in all (four out of four) well-established (Supplementary Fig. 2c) melanoma cell lines, suggesting that extended *in vitro* passage might induce MDM2 expression.

We determined TP53, BRAF and NRAS mutational status in the primary tumors and cell lines described above (Supplementary Tables 2 and 3). Consistent with previous reports<sup>15,25</sup>, TP53 mutations





were rare (Supplementary Tables 2 and 3). Notably, we found that MDM4 was overexpressed at comparable frequencies in tumors and cell lines harboring either wild-type or mutated *NRAS* or *BRAF* (Supplementary Tables 2 and 3).

### MDM4 promotes melanoma in mice

Constitutive expression of an activated form of human *NRAS* (*NRAS<sup>Q61K</sup>*) in melanocytes leads to formation of aggressive melanomas in *Tyr::NRAS<sup>Q61K</sup>* transgenic mice (mice that carry one copy of the *NRAS<sup>Q61K</sup>* transgene)<sup>26</sup>, however, melanomas develop only after a long latency and with low penetrance. To assess the role of p53 in these mice, we inactivated *Trp53* in previously characterized null (–) and conditional (*lox*) knockout alleles<sup>27,28</sup>. Melanocyte-specific deletion of one or two alleles of *Trp53* cooperated with *NRAS<sup>Q61K</sup>* to induce spontaneous and aggressive melanoma formation (ref. 29 and Supplementary Fig. 3). Although indicative of p53's role as a barrier to melanomagenesis *in vivo*, these experiments do not recapitulate the genetic makeup of human melanomas, most of which express wild-type p53 (ref. 15).

We reasoned that MDM4 could compromise p53 function *in vivo* to promote melanoma formation. We therefore induced Mdm4 overexpression in melanocytes of *Tyr::NRAS<sup>Q61K</sup>* and *Tyr::Cre<sup>fl</sup>*; *Trp53<sup>lox/+</sup>* mice by crossing these mice with the previously described Mdm4 conditional transgenic mice *ROSA26-pCAGGS-loxP-STOP-loxP-Mdm4<sup>fl</sup>* (mice carrying one copy of an Mdm4 conditional transgenic allele, referred to as *Mdm4<sup>fl</sup>*)<sup>30</sup> (Fig. 2a). We crossed these double-transgenic mice onto a *Trp53*-heterozygous background to facilitate spontaneous tumor development. As expected from the *Trp53* heterozygosity of *Tyr::NRAS<sup>Q61K</sup>; Tyr::Cre<sup>fl</sup>; Trp53<sup>+/+</sup>; Mdm4<sup>fl</sup>* mice, some mice developed T cell lymphomas and sarcomas<sup>27</sup> (data not shown). However, the vast majority of mice developed melanoma before developing other tumors (data not shown). We restricted further analyses to mice that were lymphoma- and sarcoma-free and that developed melanomas at least 3-mm thick. Quadruple-transgenic mice (*Tyr::NRAS<sup>Q61K</sup>; Tyr::Cre<sup>fl</sup>; Trp53<sup>+/+</sup>; Mdm4<sup>fl</sup>*) developed at least one melanoma significantly faster than controls (*Tyr::NRAS<sup>Q61K</sup>; Tyr::Cre<sup>fl</sup>; Trp53<sup>+/+</sup>*) (Fig. 2b). Melanomas were nodular, pigmented and

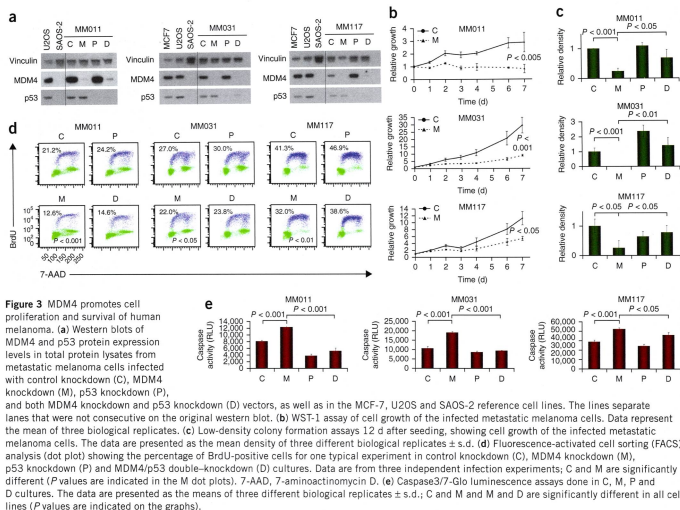


**Figure 3** MDM4 promotes cell proliferation and survival of human melanoma. (a) Western blots of MDM4 and p53 protein expression levels in total protein lysates from metastatic melanoma cells infected with control knockdown (C), MDM4 knockdown (M), p53 knockdown (P), and both MDM4 knockdown and p53 knockdown (D) vectors, as well as in the MCF-7, U2OS and SAOS-2 reference cell lines. The lines separate lanes that were not consecutive on the original western blot. (b) WST-1 assay of cell growth of the infected metastatic melanoma cells. Data represent the mean of three biological replicates. (c) Low-density colony formation assays 12 d after seeding, showing cell growth of the infected metastatic melanoma cells. The data are presented as the mean density of three different biological replicates  $\pm$  s.d. (d) Fluorescence-activated cell sorting (FACS) analysis (dot plot) showing the percentage of BrdU-positive cells for one typical experiment in control knockdown (C), MDM4 knockdown (M), p53 knockdown (P) and MDM4/p53 double-knockdown (D) cultures. Data are from three independent infection experiments; C and M are significantly different ( $P$  values are indicated in the M dot plots). 7-AAD, 7-aminoactinomycin D. (e) Caspase3/7-Glo luminescence assays done in C, M, P and D cultures. The data are presented as the means of three different biological replicates  $\pm$  s.d.; C and M and M and D are significantly different in all cell lines ( $P$  values are indicated on the graphs).

positive for S-100 protein expression<sup>31</sup>, regardless of genotype (Fig. 2c). Tumors arising in *Tyr::NRAS*<sup>Q61/p</sup>; *Tyr::Cre*<sup>fl</sup>; *Mdm4*<sup>fl</sup> mice retained high exogenous (myc-tagged) *Mdm4* expression (Fig. 2d,e). Exogenous *Mdm4* mRNA and protein expression levels were perfectly correlated.

We next assessed whether *Mdm4* overexpression also enhances melanoma development on a wild-type *Trp53* background. *Tyr::NRAS*<sup>Q61/p</sup>; *Tyr::Cre*<sup>fl</sup>; *Mdm4*<sup>fl</sup> had a nearly 100% incidence of melanoma with a much shorter average latency period than controls (*Tyr::NRAS*<sup>Q61/p</sup>; *Tyr::Cre*<sup>fl</sup>; *o/p*) (Fig. 2f). On average, there were more lesions per mouse in *Mdm4*-overexpressing mice than in controls (Fig. 2g). Together, these data indicate that *Mdm4* overexpression cooperates with the *NRAS* oncoprotein to promote aggressive melanoma formation *in vivo*.

#### MDM4 promotes cell proliferation and survival of human melanoma

To test whether human metastatic melanoma cells depend on high MDM4 expression for their survival and proliferation we knocked down MDM4 in short-term cultured (MM011, MM031, MM117) and commonly used metastatic melanoma cell lines (A375, Lu1205). Our choice of cell lines was motivated by the presence of high protein expression levels of MDM4, a genetically intact *TP53* pathway and different ratios of MDM4/MDM2 protein expression levels. We targeted different regions of MDM4 mRNA using two lentiviral

constructs (data shown below and in Supplementary Fig. 4). MM031 expressed high MDM4 and low MDM2 protein expression levels, which best represents the vast majority of melanoma lesions; MM011 and MM117 highly express both MDM4 and MDM2, which is characteristic of only a few human distant-metastatic melanomas. Western blotting confirmed efficient MDM4 knockdown in all cell lines (Fig. 3a and Supplementary Fig. 5a). Cell viability and colony formation assays showed that MDM4 knockdown caused robust growth inhibition in all melanoma lines analyzed (Fig. 3b,c and Supplementary Fig. 5b,c).

Cell cycle analysis showed a reproducible and significant decrease in BrdU incorporation upon MDM4 knockdown (Fig. 3d and Supplementary Fig. 5d). In addition to the reduced proliferation, increased cell death was evidenced by the increased sub-G1 fraction in the FACS profiles (data not shown). We found that cell death resulted, at least in part, from a reproducible and significant induction of caspase3/7 activity (Fig. 3e and Supplementary Fig. 5).

Some of the parental melanoma cell lines (MM031, Lu1205, A375) efficiently formed tumors when injected into nude mice (8-week-old Rj;NMRI-nu (nu/nu) female mice). We show that MDM4 knockdown strongly reduces the transforming potential of these cells *in vivo* (Supplementary Figs. 5f and 6). These data indicate that MDM4 regulates the survival and proliferation of high-MDM4-expressing melanoma cells *in vitro* and *in vivo*.

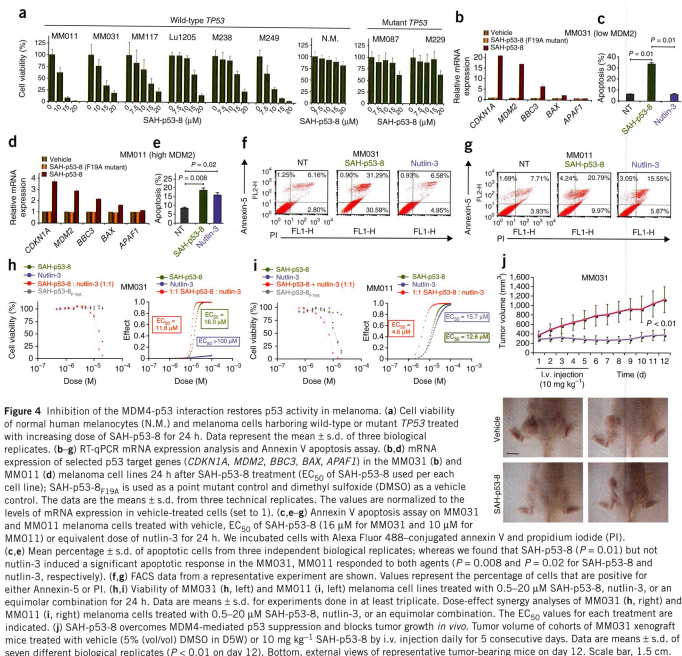


**Figure 4** Inhibition of the MDM4-p53 interaction restores p53 activity in melanoma. **(a)** Cell viability of normal human melanocytes (N.M.) and melanoma cells harboring wild-type or mutant TP53 treated with increasing doses of SAH-p53-8 for 24 h. Data represent the mean  $\pm$  s.d. of three biological replicates. **(b–g)** RT-qPCR mRNA expression analysis and Annexin V apoptosis assay. **(b,d)** mRNA expression of selected p53 target genes (*CDKN1A*, *MDM2*, *BBC3*, *BAX*, *APAF1*) in the MM031 **(b)** and MM011 **(d)** melanoma cell lines 24 h after SAH-p53-8 treatment ( $\text{EC}_{50}$  of SAH-p53-8 used per each cell line); SAH-p53-8<sub>F19A</sub> is used as a point mutant control and dimethyl sulfoxide (DMSO) as a vehicle control. The data are the means  $\pm$  s.d. from three technical replicates. The values are normalized to the levels of mRNA expression in vehicle-treated cells (set to 1). **(c,e–g)** Annexin V apoptosis assay on MM031 and MM011 melanoma cells treated with vehicle,  $\text{EC}_{50}$  of SAH-p53-8 (16  $\mu$ M for MM031 and 10  $\mu$ M for MM011) or equivalent dose of nutilin-3 for 24 h. We incubated cells with Alexa Fluor 488-conjugated Annexin V and propidium iodide (PI). **(c,e)** Mean percentage  $\pm$  s.d. of apoptotic cells from three independent biological replicates; whereas we found that SAH-p53-8 ( $P = 0.01$ ) but not nutilin-3 induced a significant apoptotic response in the MM031, MM011 responded to both agents ( $P = 0.008$  and  $P = 0.02$  for SAH-p53-8 and nutilin-3, respectively). **(f,g)** FACS data from a representative experiment are shown. Values represent the percentage of cells that are positive for either Annexin-5 or PI. **(h,i)** Viability of MM031 **(h, left)** and MM011 **(i, left)** melanoma cell lines treated with 0.5–20  $\mu$ M SAH-p53-8, nutilin-3, or an equimolar combination for 24 h. Data are means  $\pm$  s.d. for experiments done in at least triplicate. Dose-effect synergy analyses of MM031 **(h, right)** and MM011 **(i, right)** melanoma cells treated with 0.5–20  $\mu$ M SAH-p53-8, nutilin-3, or an equimolar combination. The  $\text{EC}_{50}$  values for each treatment are indicated. **(j)** SAH-p53-8 overcomes MDM4-mediated p53 suppression and blocks tumor growth *in vivo*. Tumor volume of cohorts of MM031 xenograft mice treated with vehicle (5% (vol/vol) DMSO in D5W) or 10  $\text{mg kg}^{-1}$  SAH-p53-8 by i.v. injection daily for 5 consecutive days. Data are means  $\pm$  s.d. of seven different biological replicates ( $P < 0.01$  on day 12). Bottom, external views of representative tumor-bearing mice on day 12. Scale bar, 1.5 cm.

### MDM4 protects melanoma against p53-induced apoptosis

To test whether p53 inactivation underlies dependency of melanoma on high MDM4 expression, we knocked down both MDM4 and p53 in short-term cultured metastatic melanoma cell lines (MM011, MM031, MM117; **Fig. 3a**). Colony formation assays indicated that the MDM4 KD-mediated growth inhibition at least partly depends on p53 (**Fig. 3c**).

The two best-characterized p53-mediated antiproliferative activities are induction of cell cycle arrest and apoptosis. Unexpectedly, p53 knockdown did not significantly rescue the proliferation of melanoma cells upon MDM4 knockdown (**Fig. 3d**). In contrast, p53 knockdown largely rescued MDM4 knockdown cells from apoptosis (**Fig. 3e**). Concomitant with apoptosis induction upon MDM4 knockdown, we observed increased recruitment of p53 to the promoters of its proapoptotic targets, BAX (encoding BCL2-associated X protein)

and APAF1 (encoding apoptotic peptidase activating factor 1), and a marked increase in their transcription (**Supplementary Fig. 7**). The data suggest that high MDM4 levels inhibit apoptosis in human melanoma cells by antagonizing p53.

### Inhibition of the MDM4-p53 interaction restores p53 activity

The reliance of melanoma cells on MDM4 for survival suggests that interfering with its interaction with p53 could restore apoptotic capability. SAH-p53-8 is a cell-penetrating, stabilized,  $\alpha$ -helical peptide<sup>32</sup> that binds with high affinity to MDM4 within its p53-binding pocket and is capable of disrupting p53-MDM4 complexes<sup>33</sup>. SAH-p53-8 has approximately 25-fold lower potency against MDM2 *in vitro*<sup>33</sup>. Unlike the MDM2-specific antagonist nutilin-3 (ref. 34), SAH-p53-8 can reactivate p53 and induce apoptosis in cancer cells that highly express MDM4 and wild-type p53 (ref. 33). To test whether direct

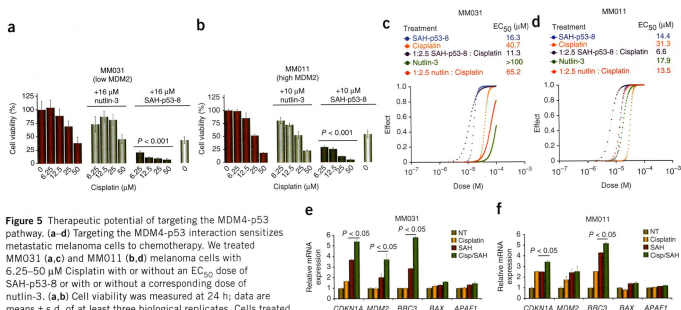


**Figure 5** Therapeutic potential of targeting the MDM4-p53 pathway. **(a–d)** Targeting the MDM4-p53 interaction sensitizes metastatic melanoma cells to chemotherapy. We treated MM031 **(a,c)** and MM011 **(b,d)** melanoma cells with 6.25–50 μM Cisplatin with or without an EC<sub>50</sub> dose of SAH-p53-8 or with or without a corresponding dose of nutlin-3. **(a,b)** Cell viability was measured at 24 h; data are means ± s.d. of at least three biological replicates. Cells treated with SAH-p53-8 show a statistically significant decrease in cell viability ( $P < 0.001$ ) compared with cells treated with Cisplatin alone. **(c,d)** Dose effect synergy analyses of MM031 **(c)** and MM011 **(d)** melanoma cells treated with 6.25–50 μM Cisplatin with or without 0.5–20 μM SAH-p53-8 or nutlin-3. **(e,f)** RT-qPCR mRNA expression analysis of selected p53 target genes in the MM031 **(e)** and MM011 **(f)** melanoma cell lines 24 h after treatment. The data represent the means ± s.d. from three technical replicates. The values are normalized to the level of mRNA expression in vehicle-treated cells (set to 1). *CDKN1A*, *MDM2* and *BBC3* mRNA expression levels differed significantly in cells treated with both Cisplatin and SAH-p53-8 compared with cells treated with the single agents ( $P < 0.05$ ) in both cell lines.

inhibition of MDM4 is a viable therapeutic strategy for melanoma, we treated a series of melanoma cell lines, including the BRAF inhibitor-sensitive melanoma M238, M249 and M229 cell lines<sup>35</sup>, with increasing concentrations of SAH-p53-8. SAH-p53-8 potentially inhibited growth of all melanoma lines carrying wild-type p53 (Fig. 4a and Supplementary Fig. 8). In contrast, melanoma cells with inactive p53 mutants did not respond to SAH-p53-8 treatment, indicating that SAH-p53-8-mediated growth inhibition depends on the presence of wild-type p53 (Fig. 4a). Moreover, SAH-p53-8 did not markedly affect normal melanocyte growth (Fig. 4a). SAH-p53-8, but not its biologically inactive point-mutant analog SAH-p53-8<sub>F19A</sub>, induces expression of p53-target genes, including *BAX*, *APAF1* and *BBC3* (also known as *PUMA*; encoding BCL2 binding component 3) in both MM031 and MM011 (Fig. 4). SAH-p53-8 induced apoptosis in all melanoma cell lines; this effect was significantly attenuated by knockdown or hypomorphic mutation of p53 (Fig. 4 and Supplementary Fig. 9).

Consistent with the fact that high MDM4 expression levels are associated with a reduced apoptotic response to MDM2 antagonists<sup>36,37</sup>, nutlin-3 induced relatively low apoptotic activity in MM011 cells despite high expression levels of MDM2 in these cells, and it induced practically no activity in MM031 cells (Fig. 4e–g).

We next treated melanoma cells with SAH-p53-8, nutlin-3 or a 1:1 stoichiometric combination of both compounds (Fig. 4h,i). As expected, nutlin-3 had only a marginal cytotoxic effect on MM031 cells. In contrast, we observed marked growth inhibition in MM031 cells in response to SAH-p53-8 alone (Fig. 4h), and we saw only a very modest increase in cytotoxicity upon combination with nutlin-3 (combination index = 0.90; combination index is calculated as described in ref. 33). MM011 cells, however, showed sensitivity to the single agents (Fig. 4i) and the two compounds synergize strongly, leading to enhanced cytotoxicity (combination index = 0.32). The mutant SAH-p53-8<sub>F19A</sub> peptide did not induce any measurable

cytotoxic effects in any of the cell lines (Fig. 4h,i). Together our data indicate that all human melanoma cell lines analyzed are either highly sensitive to MDM4 inhibition either alone or in combination with MDM2 inhibition.

To test the therapeutic potential of inhibiting the p53-MDM4 interaction *in vivo*, we evaluated the activity of SAH-p53-8 in a MM031 mouse xenograft model. Although tumor growth rate was unaffected in vehicle-treated (vehicle was 5% (vol/vol) DMSO in D5W) mice, intravenous treatment with SAH-p53-8 significantly suppressed tumor growth (Fig. 4j). As previously shown, histological examination of SAH-p53-8-treated mice indicated no obvious toxicity<sup>33</sup>. Collectively, these data show the therapeutic potential of inhibiting the MDM4-p53 interaction in melanoma.

#### SAH-p53-8 sensitizes melanoma to chemotherapy and BRAF (V600E) inhibition

DNA-damaging agents such as Cisplatin or melphalan have yielded low response rates in the treatment of melanomas<sup>38</sup>. As these agents rely partly on activating the p53 pathway, we hypothesized that co-treatment with p53-MDM4 inhibitors may enhance their potency. We therefore investigated the effects of Cisplatin and melphalan alone or in combination with either nutlin-3 or SAH-p53-8 on MM011 and MM031 cell line growth. Although treatment with these agents alone yielded variable growth inhibition, cytotoxic effects were greatly potentiated by co-treatment with SAH-p53-8 (Fig. 5a–d and Supplementary Fig. 10). Transcriptional induction of p53 targets was more robust in cells treated with both Cisplatin and SAH-p53-8 than in cells treated with single agents (Fig. 5e,f). As expected, MM031 cells expressing low levels of MDM2 responded more favorably to the combination of alkylating agents with SAH-p53-8 than to co-treatment with nutlin-3 (Fig. 5a,c). In contrast, high expression levels of MDM2 rendered MM011 cells at least partly sensitive to nutlin-3 (Fig. 5b,d); identical results were obtained with melphalan

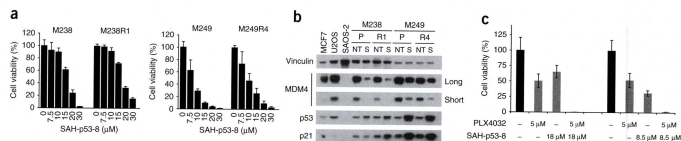


**Figure 6** Targeting the MDM4-p53 pathway sensitizes melanoma cells to a BRAF<sup>V600E</sup> inhibitor. **(a)** We exposed isogenic BRAF inhibitor-sensitive parental and BRAF inhibitor-resistant cell lines to increasing doses of SAH-p53-8 (7.5–30  $\mu$ M). We measured cell viability 24 h after SAH-p53-8 treatment. Data represent the means  $\pm$  s.d. of three biological replicates. **(b)** MDM4, p53 and p21 protein levels assessed by western blotting analysis in nontreated (NT) and SAH-p53-8 (S)-treated human melanoma cells M238, M238R1, M249 and M249R4, which were described previously<sup>35</sup>. We used MCF7, U2OS and SAOS-2 cells as reference controls; vinculin is a loading control. P; parental, R; BRAF inhibitor-resistant subline. Long and Short indicate long and short exposure, respectively. **(c)** Dose-effect synergy analyses of melanoma cells treated with 5  $\mu$ M PLX4032 and an EC<sub>50</sub> dose of SAH-p53-8 (18 and 8.5  $\mu$ M). Data are means  $\pm$  s.d. of at least three biological replicates. Response to combination treatment differed significantly from the response to single agents ( $P < 0.01$ ) in both cell lines.

(Supplementary Fig. 10). Collectively, these data suggest that targeting the MDM4-p53 interaction sensitizes melanoma cells to DNA-damaging agents.

Overcoming widespread resistance to BRAF inhibitors<sup>39</sup> is likely to require targeting of multiple signaling pathways. We investigated whether targeting the MDM4-p53 interaction could affect the growth of BRAF inhibitor-sensitive parental melanoma cell lines (M238 and M249) as well as BRAF-resistant sublines (M238R1 and M249R4) that were artificially derived by chronic exposure to a BRAF inhibitor<sup>35</sup>. All cell lines express high MDM4 protein expression levels and harbor wild-type p53. Treatment of both parental and BRAF-resistant cell lines with increasing doses of SAH-p53-8 led to decreased viability, indicating that cells that have acquired drug resistance to BRAF inhibitor remain largely sensitive to MDM4-p53 targeting (Fig. 6a). We saw a comparable increase in expression of p53 and its target p21 in the parental and resistant lines upon SAH-p53-8 exposure (Fig. 6b). Notably, SAH-p53-8 exposure led to a marked decrease in MDM4 protein expression levels (Fig. 6b).

To investigate whether combined BRAF (V600E) and MDM4 inhibition synergizes to induce cytotoxicity, we treated BRAF inhibitor-sensitive lines with a BRAF inhibitor, PLX4032, and with SAH-p53-8 individually or in combination (Fig. 6c). PLX4032 alone was sufficient to decrease the viability of these cells (Fig. 6c). Co-treatment with PLX4032 and SAH-p53-8 significantly enhanced the effect when compared with each individual compound (Fig. 6c).

## DISCUSSION

Unlike in most other tumor types, TP53 mutations are rare in melanomas, making the relevance of the p53 pathway in melanomagenesis controversial. Given our observation of MDM4 overexpression in about 65% of melanomas, we contend that MDM4 overexpression is an important oncogenic event that alters p53 function in melanoma in a large proportion of patients.

Because MDM4 overexpression is primarily detected at the protein level, its detection inevitably escaped previous investigations that focused on transcriptomic analyses. This raises a possibility that MDM4 overexpression might be an even more frequent oncogenic event than previously anticipated in other tumor types as well<sup>23</sup>.

At physiological levels MDM4 serves as a constitutive buffer against untoward p53 activity<sup>23,40,41</sup>; its oncogenic activity results from its ability to attenuate p53 function<sup>23,24</sup>. Consistent with this, we found that most melanoma cells depend on high MDM4 expression

to survive by keeping the proapoptotic functions of p53 in check. Furthermore, we found that high MDM4 expression levels are also required, in a p53-independent manner, by melanoma cells for proliferation in culture. This raises the possibility that MDM4 possesses additional oncogenic functions, independent of p53 suppression. Although p53-independent function(s) may contribute to MDM4-mediated oncogenesis, we found that targeting the MDM4-p53 interaction was sufficient to suppress the growth of metastatic melanoma cells with wild-type p53.

Restoration of p53 function has been extensively pursued as a therapeutic approach in cancers in which, as in most melanomas, p53 function is compromised although the TP53 locus remains intact<sup>42–44</sup>. Several efforts have focused on blocking MDM2 as a strategy to reactivate p53 (refs. 34,45–48), despite several caveats<sup>49</sup>. One major limitation of MDM2-specific therapy is that tumor cells in which MDM4 expression is high but MDM2 is low respond poorly to MDM2 inhibition<sup>13,33</sup>. We found that most human melanoma cell lines express high MDM4 and low MDM2 levels and indeed respond very poorly to treatment with the MDM2-specific antagonist nutlin-3. In contrast, these cells are extremely sensitive to SAH-p53-8, thus indicating that pharmacological disruption of the MDM4-p53 interaction is required to induce p53 signaling in these cells. Note, however, that SAH-p53-8 can also suppress MDM2-p53 binding to some extent. It therefore cannot be ruled out that SAH-p53-8 effectiveness against melanoma cells with a high MDM4/MDM2 ratio depends, at least partly, on MDM2 targeting. Melanomas that express high levels of both MDM4 and MDM2 are rare, although we found that cell lines with this profile were sensitive to either SAH-p53-8 or nutlin-3 but even more sensitive to combination treatment. Therefore, whereas the majority of melanoma patients may respond poorly to single MDM2 inhibitors, they could benefit greatly from treatment with single MDM4 or dual MDM4/MDM2 antagonists.

Recent data argue that MDM4 inhibition is a more feasible and less hazardous strategy than MDM2 inhibition for restoring p53 function. For instance, genetic ablation of *Mdm2* in normal adult tissues, including those that are quiescent and fully differentiated, leads to pathologies because of the induction of p53-dependent cell death<sup>20</sup>. Hence, toxicity after systemic exposure to potent MDM2 inhibitors is a serious concern. In contrast, ablation of *Mdm4* in most adult somatic tissues leads to either modest or no phenotypic consequences<sup>40,41</sup>.

Most patients with metastatic melanoma do not respond to conventional chemotherapy regimens<sup>50</sup>. Our results show that inhibition of



the MDM4-p53 interaction greatly potentiates the cytotoxic effects of two widely used chemotherapeutic agents. MDM4 inhibitors could therefore be used to enhance the effectiveness of conventional therapeutic agents in the treatment of melanoma.

Similarly, despite the initial success with BRAF inhibitors, chronic treatment is invariably associated with the development of drug resistance<sup>39</sup>. Management of BRAF inhibitor resistance is likely to be achieved through combination therapy that targets multiple pathways. We have shown that MDM4 is overexpressed in melanomas harboring mutations in BRAF and NRAS alike, that MDM4-p53 inhibition equally affects the growth of NRAS- and BRAF-mutant melanoma cells and, notably, that MDM4-p53 inhibition is equally effective at inhibiting growth of BRAF-mutant cells before or after they acquire resistance to BRAF inhibitors. We also show that combined treatment with MDM4-p53 inhibitors and BRAF inhibitors synergizes to kill melanoma cells that are sensitive to BRAF inhibition.

Our findings suggest that MDM4-p53 targeting offers a promising approach to improve the clinical benefits of BRAF inhibition. Crucially, our *in vitro* results predict that MDM4-p53 targeted therapy may be effective in cases when BRAF inhibitor resistance has already been acquired and in melanomas with NRAS mutations, for which no specific inhibitors exist. Prioritization should therefore be given to the preclinical development of strategies that inhibit MDM4-mediated suppression of p53 function in human melanoma.

## METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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## AUTHOR CONTRIBUTIONS

A.G. did experimental work, developed the hypothesis, analyzed the data and coordinated the project. F.L. did experimental work and analyzed the data. C.F. conducted immunofluorescence analyses in normal human skin and melanomas and analyzed the data. E.A.R. conducted cellular assays. M.D. conducted experimental work. S.V. determined the p53 status of melanoma cell lines and primary tumors. A.Z. did the chromatin immunoprecipitation experiments and analyzed the data. S.H. contributed to the development of MDM4 immunohistochemistry. J.L.I. generated MDM4 knockdown lentiviral vectors. D.Y. and J.G. obtained primary human melanoma samples. J.H.H. contributed to the mouse work and experimental design. H.S. and G.M. generated and characterized BRAF inhibitor-resistant cell lines. F.B. produced and supplied SAH-p53-8 and SAH-p53-F343. Y.H., L.L., A.J., R.S.L., G.G., M.S. and F.B. discussed the hypothesis and contributed to data interpretation and experimental design. J.C.M. conceived the hypothesis, led the project, interpreted the data and wrote the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# Robust tumor immunity to melanoma mediated by interleukin-9–producing T cells

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Interleukin-9 (IL-9) is a T cell cytokine that acts through a  $\gamma$ C-family receptor on target cells and is associated with inflammation and allergy. We determined that T cells from mice deficient in the T helper type 17 ( $T_H17$ ) pathway genes encoding retinoid-related orphan receptor  $\gamma$  (ROR- $\gamma$ ) and IL-23 receptor (IL-23R) produced abundant IL-9, and we found substantial growth inhibition of B16F10 melanoma in these mice. IL-9–blocking antibodies reversed this tumor growth inhibition and enhanced tumor growth in wild-type (WT) mice. *IL9r<sup>-/-</sup>* mice showed accelerated tumor growth, and administration of recombinant IL-9 (rIL-9) to tumor-bearing WT and *Rag1<sup>-/-</sup>* mice inhibited melanoma as well as lung carcinoma growth. Adoptive transfer of tumor-antigen–specific  $T_H9$  cells into both WT and *Rag1<sup>-/-</sup>* mice suppressed melanoma growth; this effect was abrogated by treatment with neutralizing antibodies to IL-9. Exogenous rIL-9 inhibited tumor growth in *Rag1<sup>-/-</sup>* mice but not in mast-cell–deficient mice, suggesting that the targets of IL-9 in this setting include mast cells but not T or B cells. In addition, we found higher numbers of  $T_H9$  cells in normal human skin and blood compared to metastatic lesions of subjects with progressive stage IV melanoma. These results suggest a role for IL-9 in tumor immunity and offer insight into potential therapeutic strategies.

Recent studies suggest that immunologic targeting of melanoma is a promising therapeutic strategy<sup>1,2</sup>. A key role for CD4<sup>+</sup> T cells in tumor immunity has emerged from several studies using mouse models<sup>3–6</sup>. Adoptive transfer of tumor-specific CD4<sup>+</sup> T cells has been shown to eradicate large established and metastatic melanomas in mice<sup>5–7</sup>. The role of  $T_H17$  cells in tumor immunity is, however, not clear, with apparently contradictory results having been published<sup>8–13</sup>. ROR- $\gamma$ , an isoform of ROR- $\gamma$ , is a lineage-specific transcription factor that is crucial for the development of  $T_H17$  cells<sup>14–16</sup> and whose deficiency abrogates the development of IL-17A–secreting  $T_H17$  cells. High expression of ROR- $\gamma$  is reported in autoimmune and inflammatory diseases<sup>14,17</sup>. We explored a distinct approach to studying the role of  $T_H17$  cells in tumor immunity using mice whose T cells were deficient in this transcription factor. Using ROR- $\gamma$ –deficient mice (*Rorc<sup>-/-</sup>* mice), we observed marked growth inhibition of subcutaneous B16F10 melanomas compared to WT mice. Transcriptional profiling experiments using purified CD4<sup>+</sup> T cells differentiated under  $T_H17$  polarizing conditions revealed strikingly high expression of IL-9 in ROR- $\gamma$  CD4<sup>+</sup> T cells compared with ROR- $\gamma$  CD4<sup>+</sup> T cells. We therefore explored a putative role of IL-9 in antitumor immunity, and found marked tumor growth inhibition mediated by IL-9 and  $T_H9$  cells. In addition, we found  $T_H9$  cells in healthy human skin and blood, as well as in metastatic lesions of subjects with advanced melanoma. We have here identified new and previously unreported roles of IL-9 and  $T_H9$  cells in tumor immunity in mice.

## RESULTS

### Deficiency of ROR- $\gamma$ is associated with inhibited melanoma growth

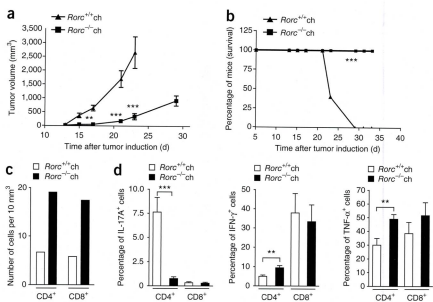
To examine the role of ROR- $\gamma$  in tumor immunity, we used a B16F10 mouse melanoma model. *Rorc<sup>-/-</sup>* mice do not develop secondary lymph nodes and have fewer CD4<sup>+</sup> and CD8<sup>+</sup> T cells than *Rorc<sup>+/+</sup>* mice<sup>18</sup>. We therefore generated ROR- $\gamma$ –deficient bone marrow chimeric mice (*Rorc<sup>-/-</sup>* ch) and wild-type chimeric mice with normal ROR- $\gamma$  expression (*Rorc<sup>+/+</sup>* ch) as a control group by administering bone marrow cells from *Rorc<sup>-/-</sup>* or *Rorc<sup>+/+</sup>* mice into sublethally irradiated *Rag1<sup>-/-</sup>* C57BL/6 mice<sup>14,16</sup>. After full reconstitution of T cells and restoration of an intact immune system (approximately 8–10 weeks after bone marrow administration), we used these chimeric mice for tumor growth experiments (Supplementary Fig. 1). We subcutaneously injected B16F10 melanoma cells into the *Rorc<sup>+/+</sup>* ch and *Rorc<sup>-/-</sup>* ch mice and monitored their tumor growth over time. Melanoma growth was strongly inhibited in the *Rorc<sup>-/-</sup>* ch mice, and the survival of the *Rorc<sup>-/-</sup>* ch mice was reproducibly longer than that of the control mice (Fig. 1a,b).

Next, we examined the infiltration of lymphocytes in tumors removed from both groups of mice. Melanomas from the *Rorc<sup>-/-</sup>* ch mice contained threefold higher numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells than melanomas from the *Rorc<sup>+/+</sup>* ch controls (Fig. 1c). T cells from the tumor draining lymph nodes of *Rorc<sup>-/-</sup>* ch melanoma-bearing mice secreted negligible amounts of IL-17A and higher amounts of interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) than the

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**Figure 1** Deficiency of ROR- $\gamma$  is associated with inhibited melanoma growth and high amounts of tumor lymphocytic infiltration. B16F10 melanoma cells were injected subcutaneously into *Rorc*<sup>-/-</sup> and *Rorc*<sup>+/+</sup> mice. (a,b) Tumor growth (a) and survival time (b) were monitored. *n* = 8 mice per group. (c) TILs were recovered and counted, as described in the Online Methods. TILs from four mice were pooled and analyzed. (d) The expression of IL-17A, IFN- $\gamma$  and TNF- $\alpha$  was analyzed by flow cytometry in CD4<sup>+</sup> and CD8<sup>+</sup> T cells in tumor draining lymph nodes. *n* = 8 mice per group. All data are mean  $\pm$  s.e.m.  $^{**}P < 0.025$ ,  $^{***}P < 0.005$  by two-tailed Student's *t* test. Two to three additional independent experiments provided similar results.



control mice (Fig. 1d). There was no difference in the number of melanoma-tumor-infiltrating CD4<sup>+</sup>CD25<sup>+</sup>, forkhead box P3 (FoxP3)<sup>+</sup> T cells (T regulatory cells) between the *Rorc*<sup>+/+</sup>ch (7.2%  $\pm$  0.4% (mean  $\pm$  s.e.m.)) and the *Rorc*<sup>-/-</sup> ch mice (5.9%  $\pm$  0.7%).

### High IL-9 expression in ROR- $\gamma$ T cells

To further explore the mechanism of melanoma growth inhibition in *Rorc*<sup>-/-</sup> mice, we performed transcriptional profiling analyses using CD4<sup>+</sup> T cells from *Rorc*<sup>-/-</sup> and *Rorc*<sup>+/+</sup> mice differentiated under T<sub>H</sub>17 polarizing conditions. The expression of IL-17A, IL-17F and IL-23R was much lower in the ROR- $\gamma$ CD4<sup>+</sup> T cells than in the ROR- $\gamma$ CD4<sup>+</sup> T cells (Fig. 2). However, expression of IL-9 in the ROR- $\gamma$ CD4<sup>+</sup> T cells was markedly higher than in the ROR- $\gamma$ CD4<sup>+</sup> T cells (Fig. 2 and Supplementary Fig. 2). The expression levels of granzyme-B and granzyme-C were also markedly elevated in ROR- $\gamma$ CD4<sup>+</sup> T cells compared to ROR- $\gamma$ CD4<sup>+</sup> T cells. Because of the substantial upregulation of IL-9 under these conditions, we focused our subsequent experiments on the potential role of IL-9 in tumor immunity.

To determine whether the T cells from the *Rorc*<sup>-/-</sup> mice had an intrinsic property that predisposed them to IL-9 production, we examined CD4<sup>+</sup> T cells from the *Rorc*<sup>-/-</sup> mice. We found higher expression of IL-9 in ROR- $\gamma$ CD4<sup>+</sup> T cells (Supplementary Fig. 3a,b) and in memory ROR- $\gamma$ CD4<sup>+</sup> T cells than in the corresponding ROR- $\gamma$ CD4<sup>+</sup> T cells and memory ROR- $\gamma$ CD4<sup>+</sup> T cells (Supplementary Fig. 3c-e). However, we observed negligible IL-9 expression in naive CD4<sup>+</sup> T cells, and crucially, there was no difference in IL-9 expression between ROR- $\gamma$ <sup>+</sup> and ROR- $\gamma$ <sup>-</sup> naive CD4<sup>+</sup> T cells (Supplementary Fig. 3f), suggesting that naive ROR- $\gamma$ <sup>+</sup> T cells have no intrinsic propensity toward IL-9 production (Supplementary Fig. 3f). T<sub>H</sub>2 and T<sub>H</sub>9 cells both secrete IL-9 (refs. 19,20), and ROR- $\gamma$ T<sub>H</sub>2 cells had higher IL-9 expression than ROR- $\gamma$ <sup>+</sup>T<sub>H</sub>2 cells. However, ROR- $\gamma$ CD4<sup>+</sup> T cells polarized under T<sub>H</sub>9 conditions showed comparable IL-9 production to ROR- $\gamma$ <sup>+</sup>T<sub>H</sub>9 cells (Supplementary Fig. 3g,h).

To determine whether the melanoma growth-inhibitory response in the *Rorc*<sup>-/-</sup> mice could be attributed to IL-9, we subcutaneously injected melanoma cells into *Rorc*<sup>-/-</sup> mice as well as *Rorc*<sup>+/+</sup> mice. The tumor growth in the *Rorc*<sup>-/-</sup> mice lagged well behind tumor growth in the *Rorc*<sup>+/+</sup> mice (Fig. 3a). We then treated tumor-bearing *Rorc*<sup>-/-</sup> mice with neutralizing antibodies to IL-9. The impaired melanoma growth in the *Rorc*<sup>-/-</sup> mice was substantially, but not

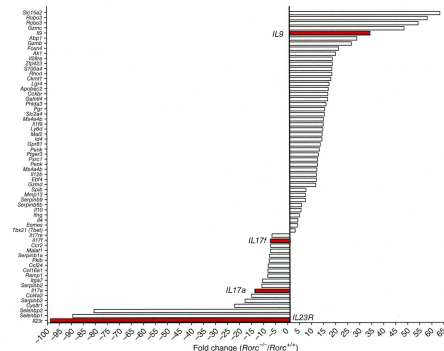
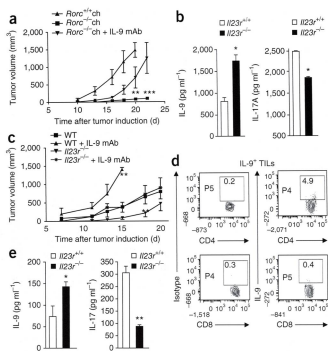


**Figure 2** High IL-9 expression in ROR- $\gamma$  T cells. Sorted naive T<sub>H</sub> cells (CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>high</sup> cells) from *Rorc*<sup>+/+</sup> and *Rorc*<sup>-/-</sup> mice were differentiated under T<sub>H</sub>17 polarizing conditions. After 4 d of exposure to T<sub>H</sub>17 polarizing conditions, the cells were harvested for transcriptional profiling experiments. A gene expression analysis was performed, and the expression of a set of genes is shown as a fold change between the *Rorc*<sup>-/-</sup> and *Rorc*<sup>+/+</sup> mice. Two additional microarray analyses provided similar results.



completely, reversed by neutralization of IL-9 (Fig. 3a). Therefore, we conclude that the melanoma growth inhibition observed in the *Rorc*<sup>-/-</sup> ch mice was partially dependent on IL-9.

### The role of IL-9 in melanoma immunity in *Il23r*<sup>-/-</sup> mice

The most strongly downregulated gene in the transcriptional profiling of *ROR*<sup>γ</sup> CD4<sup>+</sup> T cells was *Il23r* (Fig. 2). We therefore explored melanoma tumor growth in *Il23r*<sup>-/-</sup> mice. We differentiated naive CD4<sup>+</sup> T cells from *Il23r*<sup>+/+</sup> and *Il23r*<sup>-/-</sup> mice under *T*<sub>H</sub>17 polarizing conditions and re-stimulated these cells using monoclonal antibodies (mAbs) to CD3 and CD28. At 48 h after re-stimulation, we collected the supernatant and measured the amounts of IL-9 and IL-17 (ref. 19). We found that the IL-23R<sup>+</sup> CD4<sup>+</sup> T cells produced less IL-17A than did the IL-23R<sup>+</sup> CD4<sup>+</sup> T cells (Fig. 3b). However, the IL-23R<sup>+</sup> CD4<sup>+</sup> T cells showed higher IL-9 expression than the IL-23R<sup>+</sup> CD4<sup>+</sup> T cells (Fig. 3b).

We then injected melanoma cells into *Il23r*<sup>+/+</sup> and *Il23r*<sup>-/-</sup> mice and found that melanoma growth was greatly impaired in the *Il23r*<sup>-/-</sup> mice. To investigate the role of IL-9 in this tumor growth inhibition, we treated these mice with a neutralizing antibody to IL-9. This neutralization of IL-9 led to enhanced melanoma tumor growth in the *Il23r*<sup>-/-</sup> mice (Fig. 3c), suggesting that the production of IL-9 by tumor-infiltrating lymphocytes (TILs) (Fig. 3d) had a role in suppressing tumor growth. In addition, we isolated and stimulated tumor-site draining lymph node cells (LNCs) from *Il23r*<sup>+/+</sup> and *Il23r*<sup>-/-</sup> mice using mAbs specific to CD3 and CD28 for 48 h. We found that IL-23R<sup>+</sup> T cells secreted more IL-9 *in vitro* than IL-23R<sup>+</sup> T cells (Fig. 3e).

### *T*<sub>H</sub>9 cells inhibit melanoma growth

As IL-9 is produced mainly by CD4<sup>+</sup> T cells<sup>20,21</sup>, we examined the role of subsets

**Figure 3** The role of IL-9 in melanoma immunity in *Il23r*<sup>-/-</sup> mice.

(a) Tumor volume in control (*Rorc*<sup>+/+</sup> ch) and *Rorc*<sup>-/-</sup> ch mice after subcutaneous injection of B16F10 melanoma cells in both groups and administration of neutralizing antibody to IL-9 in the *Rorc*<sup>-/-</sup> group. (b) IL-9 and IL-17A concentrations estimated by ELISA of re-stimulated *in vitro*-differentiated *T*<sub>H</sub>17 cells from *Il23r*<sup>+/+</sup> and *Il23r*<sup>-/-</sup> mice. (c) Tumor volumes over time in *Il23r*<sup>-/-</sup> and control *Il23r*<sup>+/+</sup> mice after subcutaneous injection of B16F10 melanoma cells and administration of neutralizing antibody to IL-9 to both groups. (d) Percentage of IL-9-producing TILs (indicated in the boxes) isolated from tumors growing in WT mice after staining and quantification by flow cytometry. (e) IL-9 and IL-17 concentrations as estimated by ELISA in supernatants from tumor draining LNCs. All data are mean ± s.e.m. \**P* < 0.05, \*\**P* < 0.025, \*\*\**P* < 0.005 by two-tailed Student's *t* test. Two additional independent experiments (a,c) produced similar results.

of CD4<sup>+</sup> effector T cells in melanoma growth in mice. We generated *T*<sub>H</sub>1, *T*<sub>H</sub>2, *T*<sub>H</sub>9 and *T*<sub>H</sub>17 cells from naive CD4<sup>+</sup> T cells of OT-II mice. CD4<sup>+</sup> T cells from OT-II mice express a transgenic T cell receptor that recognizes residues 323–339 from ovalbumin in the context of a mouse major histocompatibility (MHC) class II allele expressed in B6 mice (I-A<sup>b</sup>). B16F10-ova melanoma cells express the full-length ovalbumin molecule. Before their adoptive transfer into mice, we analyzed the *in vitro* polarized CD4<sup>+</sup> T cells for their cytokine expression (Supplementary Fig. 4). We then transferred the differentiated *T*<sub>H</sub> cells and subcutaneously injected B16F10-ova melanoma cells into syngeneic immunocompetent WT mice. The mice treated with the *T*<sub>H</sub>9 cells showed the greatest resistance to melanoma growth compared to mice treated with all other *T*<sub>H</sub> cell subsets (Fig. 4a).

To determine whether *T*<sub>H</sub>9 cells could inhibit the growth of melanoma independently of endogenous T cells, we transferred *T*<sub>H</sub>0 (stimulated and expanded *in vitro* but not polarized by exogenous cytokines) and *T*<sub>H</sub>9 cells generated from OT-II mice into immunocompromised host mice (*Rag1*<sup>-/-</sup>). In this setting, the *T*<sub>H</sub>9 cells inhibited the growth of subcutaneous melanoma tumors (Fig. 4b), and the survival of the *T*<sub>H</sub>9-treated mice was markedly longer than that of the *T*<sub>H</sub>0-treated mice (Fig. 4c). These data suggest that *T*<sub>H</sub>9 cells are capable of inhibiting melanoma growth, even in the absence of CD8<sup>+</sup> T cells. Moreover, blockade of IL-9 accelerated melanoma growth in the *T*<sub>H</sub>9-treated mice, suggesting a partial dependence of this effect on IL-9 (Fig. 4b).

To determine whether *T*<sub>H</sub>9 cells could directly kill melanoma cells, we cocultured carboxyfluorescein succinimidyl ester (CFSE) labeled B16F10-ova cells with *in vitro*-generated *T*<sub>H</sub>0, *T*<sub>H</sub>9 and *T*<sub>H</sub>17 cells from OT-II mice for 24 h. After 24 h of culture, we analyzed apoptosis of tumor cells by staining the B16F10-ova cells

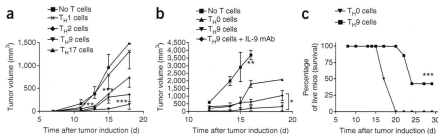


**Figure 4** *T*<sub>H</sub>9 cells inhibit melanoma growth. (a–c) Tumor volume over time after the generation of differentiated *T*<sub>H</sub> cells from OT-II mice and the transfer of these cells into wild-type (WT) C57BL/6 mice (a) or *Rag1*<sup>-/-</sup> C57BL/6 mice (b,c), with subcutaneous injection of B16F10-ova cells on the same day, *n* = 6 mice per group. In addition, *T*<sub>H</sub>9-treated mice were also given neutralizing antibodies to IL-9 or isotype control antibodies (b). All data are mean ± s.e.m. \**P* < 0.05, \*\**P* < 0.025, \*\*\**P* < 0.005 by two-tailed Student's *t* test. The significant differences in a are compared to the 'no T cell' group.



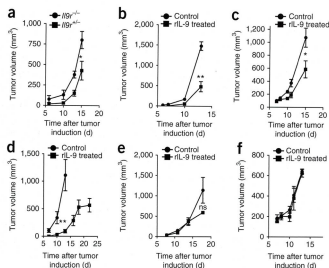


**Figure 5** Abrogation of IL-9-IL-9R signaling promotes, and treatment with rIL-9 inhibits melanoma development. (a–f) Tumor volumes over time in mice injected with either B16F10 melanoma cells or LLC cells. (a,b,d,e) Tumor volumes over time in *IL9r<sup>-/-</sup>* and control (*IL9r<sup>+/+</sup>*) mice (a), WT mice (b), *Rag1<sup>-/-</sup>* mice (d) and mast-cell-deficient mice (e) after subcutaneous injection of B16F10 melanoma cells. (c,f) Tumor volumes over time in WT mice (c) and mast-cell-deficient mice (f) after subcutaneous injection of LLC cells. Where indicated, rIL-9 was also administered, and control mice received PBS. All data are mean  $\pm$  s.e.m.  $n = 4$  mice per group for a–f. \* $P < 0.05$ , \*\* $P < 0.025$  by two-tailed Student's  $t$  test. Two to four additional independent experiments produced similar results.

with 7-aminoactinomycin D (7-AAD). The  $T_H9$  cells were capable of inducing apoptosis in the melanoma cells, whereas the  $T_H0$  and  $T_H17$  cells were much less effective in this regard (Supplementary Fig. 5a). We then assessed the mechanism of the direct cytotoxicity of the  $T_H9$  cells by measuring a limited profile of effector molecules in  $T_H9$  cells (Supplementary Fig. 5b). We observed high expression of granzyme-B in the  $T_H9$  cells, and inhibition of granzyme-B expression by a granzyme-B-specific inhibitor (5  $\mu$ M, ZAAD-CMK, Enzo Life Sciences, USA) diminished the cytotoxic activity of the  $T_H9$  cells against B16F10 melanoma cells (Supplementary Fig. 5c). To further explore the direct cytotoxic effects of  $T_H9$  cells, we incubated  $T_H9$  cells from OT-II mice with CFSE-labeled B16F10-ova cells or CFSE-labeled EL4 T lymphoma cells for 36 h. The  $T_H9$  cells had dose-dependent effects on B16F10-ova tumor cell lysis (Supplementary Fig. 6c). However, the OT-II  $T_H9$  cells did not kill the EL4 cells, which do not express ovalbumin (Supplementary Fig. 6c).

### Treatment with exogenous rIL-9 inhibits tumor growth

To analyze the role of IL-9 signaling in tumor growth, we injected B16F10 melanoma cells into *IL9r<sup>-/-</sup>* mice and *IL9r<sup>+/+</sup>* mice (control group) and monitored tumor growth. Melanoma tumor growth was accelerated in the *IL9r<sup>-/-</sup>* mice compared to growth in the *IL9r<sup>+/+</sup>* mice (Fig. 5a). We next asked whether administration of rIL-9 could protect mice against melanoma growth and progression. Remarkably, treatment

of melanoma-bearing WT mice with rIL-9 both impaired melanoma growth (Fig. 5b) and increased the survival of the WT mice compared to the untreated group (data not shown). To determine whether rIL-9 could potentiate the anti-tumor response of a melanoma vaccine, we vaccinated mice with  $10^6$  irradiated melanoma cells. We then injected live B16F10 melanoma cells 7 d later into all vaccinated mice and divided these mice into two groups. We then treated one group of mice with rIL-9 and another group served as controls (untreated). The rIL-9-treated mice showed markedly less melanoma tumor growth than did the untreated control mice (Supplementary Fig. 6a).

We then investigated whether rIL-9 could inhibit an unrelated tumor, Lewis lung carcinoma (LLC). The growth of LLC-1 tumor cells was substantially suppressed in rIL-9-treated mice compared to the untreated control mice, showing that the antitumor effect of rIL-9 was not limited to melanoma (Fig. 5c). However, rIL-9 had no effect on the growth of EL4 lymphomas (data not shown). To rule out the possibility of a direct effect of IL-9 on the tumor cells, we studied IL-9R expression in tumor cells and examined the direct effects of IL-9 on tumor cell growth (Supplementary Fig. 6b–d). B16F10 cells and LLC-1 cells showed negligible IL-9R expression, and IL-9 did not affect the growth of these cells *in vitro*, suggesting that the IL-9-mediated antitumor effects on melanoma cells and lung carcinoma cells are indirect. However, we did observe IL-9R expression in EL4 cells (Supplementary Fig. 6c), and we speculate that a direct survival-promoting effect of IL-9 may have protected the EL4 cells from the indirect antitumor effects we observed in the B16F10 and LLC-1 cells.

### Mast cells are required for the antitumor effect of rIL-9

To investigate the mechanism of IL-9-mediated tumor growth inhibition, we injected B16F10 melanoma cells into *Rag1<sup>-/-</sup>* C57BL/6 mice, which lack T and B cells, and treated the mice with rIL-9. Surprisingly, treatment with rIL-9 inhibited melanoma growth (Fig. 5d), suggesting

**Figure 6** The presence of  $T_H9$  cells in human skin and peripheral blood. (a) Skin-homing (cutaneous lymphocyte-associated antigen (CLA)<sup>+</sup>) memory T cells of peripheral blood (PBMCs) and skin-resident T cells from a healthy donor after staining for IL-9, IFN- $\gamma$ , IL-4 and IL-17 and analysis by flow cytometry. The number in each quadrant indicates the percentage of cytokine-positive cells. (b) The percentage of  $T_H9$  cells in populations of memory T cells isolated from healthy peripheral blood ( $n = 3$ ), healthy adult skin ( $n = 8$ ) and metastatic melanoma lesions ( $n = 8$ ). (c) IL-9 production as measured in cell-free supernatants by Luminex assay from memory T cells of peripheral blood (PBMCs) from healthy donors, skin T cells from healthy donors and TILs of subjects with metastatic melanoma. All data are mean  $\pm$  s.e.m.  $n = 8$  (skin-resident T cells),  $n = 3$  (PBMCs),  $n = 8$  (metastatic melanoma).

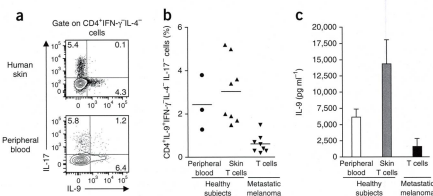


**Figure 6** The presence of  $T_H9$  cells in human skin and peripheral blood. (a) Skin-homing (cutaneous lymphocyte-associated antigen (CLA)<sup>+</sup>) memory T cells of peripheral blood (PBMCs) and skin-resident T cells from a healthy donor after staining for IL-9, IFN- $\gamma$ , IL-4 and IL-17 and analysis by flow cytometry. The number in each quadrant indicates the percentage of cytokine-positive cells. (b) The percentage of  $T_H9$  cells in populations of memory T cells isolated from healthy peripheral blood ( $n = 3$ ), healthy adult skin ( $n = 8$ ) and metastatic melanoma lesions ( $n = 8$ ). (c) IL-9 production as measured in cell-free supernatants by Luminex assay from memory T cells of peripheral blood (PBMCs) from healthy donors, skin T cells from healthy donors and TILs of subjects with metastatic melanoma. All data are mean  $\pm$  s.e.m.  $n = 8$  (skin-resident T cells),  $n = 3$  (PBMCs),  $n = 8$  (metastatic melanoma).

that the target of the effect of rIL-9 in this setting was not T or B cells. Because IL-9 is also known to promote mast-cell development and function, we injected B16F10 melanoma cells and LLC-1 cells into mast-cell-deficient (Kit W-sh/HNhrJaeBsmJ) mice and then treated the mice with rIL-9 and measured tumor growth (Fig. 5e,f). Notably, under these conditions, there was no statistically significant difference in tumor development between the rIL-9-treated and the nontreated control groups. These data suggest that the tumor growth inhibition that is mediated by the administration of rIL-9 depends on the presence of mast cells but not on the presence of T or B cells.

### T<sub>H</sub>9 cells in human skin, blood and metastatic melanoma

We next explored whether T<sub>H</sub>9 cells could be identified in humans and, if so, whether they showed a phenotype that was comparable to that of mouse T<sub>H</sub>9 cells. Mouse T<sub>H</sub>9 cells are defined as a distinct population of CD4<sup>+</sup> T cells that produce IL-9 but not IFN- $\gamma$ , IL-4 or IL-17. We found that human memory T cells isolated from peripheral blood contained a distinct population of IL-9-producing cells that do not produce IFN- $\gamma$ , IL-4 or IL-17. We also found T cells with an identical phenotype that were present in even greater abundance in resident T cells (T<sub>RM</sub>) isolated from healthy human skin (Fig. 6a,b). We then examined TILs extracted from metastatic lesions of patients with stage 4 metastatic melanoma (with metastasis to the lung ( $n = 4$ ), skin ( $n = 2$ ), adrenal gland ( $n = 1$ ) or bone ( $n = 1$ )) for the presence of IL-9-producing T cells. We observed detectable IL-9-producing T cells in six out of the eight melanoma biopsies; however, T<sub>H</sub>9 cells were less abundant in the TILs from these melanoma lesions compared to skin and blood of healthy donors, and the production of IL-9 was demonstrably lower in the T cells from the metastatic melanoma than in memory T cell populations of skin and blood from healthy donors (Fig. 6c).

### DISCUSSION

In this study, we report that mice deficient in pathways related to T<sub>H</sub>17 cell development (*Rorc*<sup>-/-</sup> and *Il23r*<sup>-/-</sup> mice) have substantial resistance to melanoma growth in a manner that is dependent largely on IL-9. In addition, T<sub>H</sub>9 cells inhibit tumor growth in an IL-9-dependent manner. Treatment of tumor-bearing mice with exogenous rIL-9 inhibits tumor growth, and this effect requires the presence of mast cells but not T or B cells. We also show that memory T<sub>H</sub>9 cells are present in healthy human blood and skin and are also present, albeit at lower amounts, in metastatic melanoma lesions compared with healthy skin and blood. To our knowledge, these findings have not been previously reported.

At the outset of the study, our goal was to assess the role of T<sub>H</sub>17 cells in melanoma immunity; thus, we used *Rorc*<sup>-/-</sup> ch mice and *Il23r*<sup>-/-</sup> mice and found enhanced tumor immunity and delayed tumor growth in both models, suggesting that an intact T<sub>H</sub>17 pathway favored tumor growth. It was not clear, however, whether blocking the T<sub>H</sub>17 pathway may have favored the emergence of a previously unappreciated antitumor pathway. To explore this possibility, we performed a transcriptional profiling analysis of ROR- $\gamma$  CD4<sup>+</sup> T cells, which revealed notably higher expression of IL-9 in these cells than in ROR- $\gamma$  CD4<sup>+</sup> T cells. Little is currently known about the regulation of IL-9. Recent studies have suggested that IL-25 and IL-21 enhance IL-9 expression in mice and humans, respectively<sup>22,23</sup>. We do not know the precise mechanism by which the absence of ROR- $\gamma$  promotes IL-9 expression in T cells, although an analysis of the IL-9 promoter did not reveal an ROR- $\gamma$  binding site (V.K.K., unpublished observations). Our data show greatly impaired IL-23R expression in ROR- $\gamma$  CD4<sup>+</sup> T cells, as well as higher IL-9 expression in IL-23R CD4<sup>+</sup> T cells than

in IL-23R<sup>+</sup> CD4<sup>+</sup> T cells. Therefore, inhibition of signaling by reduced IL-23R expression in ROR- $\gamma$  CD4<sup>+</sup> T cells might be one of the factors responsible for the higher IL-9 expression.

As IL-9 is a product primarily of CD4<sup>+</sup> T cells, we explored the role of T<sub>H</sub>9 cells in tumor immunity. Mice treated with adoptively transferred T<sub>H</sub>9 cells showed profound resistance to melanoma growth. To our knowledge, this is the first report showing an antitumor effect of T<sub>H</sub>9 cells. Previous studies have reported that the adoptive transfer of T<sub>H</sub>9 cells can induce colitis and peripheral neuritis in lymphopenic hosts<sup>21</sup>, which is consistent with the key immune effector functions that are mediated by these cells. Similar to previous reports<sup>7,9,24</sup>, in our experiments, T<sub>H</sub>2 and T<sub>H</sub>17 cells, but not T<sub>H</sub>1 or T<sub>H</sub>1 cells, inhibited melanoma growth. However, the melanoma growth-inhibitory properties of the T<sub>H</sub>9 cells were superior to those of the T<sub>H</sub>17 cells.

To determine independently whether IL-9 has melanoma growth-inhibitory properties, we studied tumor growth in *Il9r*<sup>-/-</sup> mice and found that the melanoma tumor growth was reproducibly enhanced in these mice. In addition, treatment with exogenous IL-9 reproducibly suppressed the growth of B16F10 melanoma and LLC-1 cells but not of EL4 cells in WT mice. Next, we examined the IL-9R expression on tumor cells (B16F10 melanoma cells, LLC-1 lewis lung carcinoma cells and EL4 lymphoma cells) and observed expression of IL-9R only on EL4 lymphoma cells. This suggests that the presence of IL-9R in EL4 cells, and the previously reported growth- and survival-promoting properties of IL-9 on T cells, may be responsible for the negligible antitumor effect of IL-9 in this tumor model<sup>25</sup>. Therefore, these data indicate that potential tumor targets of rIL-9 therapy should be selected with care; specifically, lymphomas and other cells known to express receptors for this cytokine may not be appropriate candidates for treatment, although this issue is hypothetical and clearly requires further study. However, taken together, these data are consistent with the idea that IL-9 clearly has an inhibitory effect on solid tumor growth.

Notably, we found no difference between normal wild-type and *Rag1*<sup>-/-</sup> mice in the degree of tumor inhibition that was mediated by exogenous IL-9, suggesting that the beneficial effects of exogenous IL-9 are mediated by other immune cells. Both human<sup>26</sup> and mouse<sup>27</sup> mast cells are well-known targets of IL-9. Recently, mast cells have been implicated as having antitumor activity<sup>28</sup>. Indeed, we observed that treatment with rIL-9 had no measureable inhibitory effect on tumor growth in mast cell-deficient mice, suggesting that these cells have a key, although uncharacterized, role in IL-9-mediated antitumor activity. Studies are currently under way to delineate how IL-9 modulates the antitumor activity (survival and function) of mast cells.

There has been some debate as to whether T<sub>H</sub>9 cells are only present in mice, and their relevance to human disease has been questioned. However, we readily detected candidate T<sub>H</sub>9 cells in populations of human skin-resident memory T cells, as well as in the memory T cell populations in human peripheral blood mononuclear cells (PBMCs). We also found memory T<sub>H</sub>9 cells in metastatic lesions of human patients with melanoma, but at a lower abundance than the steady state of memory T<sub>H</sub>9 cells in normal human skin and blood. Our finding of low amounts of T<sub>H</sub>9 cells in human metastatic melanoma is noteworthy but should be interpreted with caution. It may be that T<sub>H</sub>9 cells are part of the normal human immune response to melanoma, and therefore augmenting their activity or providing additional IL-9 may be therapeutically advantageous in this setting. Further experiments will be necessary to assess the role of IL-9 in human cancer therapy.

The role of IL-9 in melanoma tumor immunity has not been previously studied, to our knowledge. Interestingly, however, single nucleotide polymorphisms in the IL-9 gene were previously found to be

associated with an increased risk of cutaneous malignant melanoma<sup>29</sup>. The role of IL-9 in tumor immunity in general is not clear, with at least one recent study suggesting that IL-9 promotes regulatory T cell function and inhibits tumor immunity<sup>30</sup>. In our experiments, however, blockade of endogenous IL-9 invariably accelerated tumor development in the melanoma model, and the absence of IL-9 signaling (in *IL9r<sup>-/-</sup>* mice) also enhanced melanoma growth. Although our collective results in two different tumor models are consistent with a distinct antitumor effect of IL-9, further studies will determine how generalizable these findings are to tumor growth and immunity.

Our findings suggest that strategies that favor the generation of IL-9-mediated immune responses may have an important role in the treatment of melanoma and other solid tumors. Other  $\gamma$ C cytokines, such as IL-2, IL-15 and IL-21 (refs. 31–33), have been used in the treatment of human melanoma. In addition, the adoptive therapy of melanoma-antigen-specific T cells, including TILs, has been used extensively in patients with metastatic melanoma<sup>9,34–38</sup>. Our data suggest that IL-9 and T<sub>H</sub>9 cells may also have role in the treatment of this challenging malignancy.

## METHODS

Methods and any associated references are available in the online version of the paper.

**Accession codes.** The microarray data are deposited in Gene Expression Omnibus under accession code GSE38704.

*Note: Supplementary information is available in the online version of the paper.*

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## AUTHOR CONTRIBUTIONS

R.P. designed the study, performed and analyzed the experiments, and wrote the manuscript. S.X. and H.S.K. performed experiments. W.E., A.M.J., S.J.K. and V.K.K. discussed the data, provided reagents and edited the manuscript. X.J. provided reagents. C.S., R.A.C. and R.C.F. performed the human T cell experiments, discussed the data and edited the manuscript. T.S.K. designed the study, analyzed the experiments and wrote the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival

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IMA901 is the first therapeutic vaccine for renal cell cancer (RCC) consisting of multiple tumor-associated peptides (TUMAPs) confirmed to be naturally presented in human cancer tissue. We treated a total of 96 human leukocyte antigen A (HLA-A)\*02+ subjects with advanced RCC with IMA901 in two consecutive studies. In the phase 1 study, the T cell responses of the patients to multiple TUMAPs were associated with better disease control and lower numbers of prevaccine forkhead box P3 (FOXP3)+ regulatory T (T<sub>reg</sub>) cells. The randomized phase 2 trial showed that a single dose of cyclophosphamide reduced the number of T<sub>reg</sub> cells and confirmed that immune responses to multiple TUMAPs were associated with longer overall survival. Furthermore, among six predefined populations of myeloid-derived suppressor cells, two were prognostic for overall survival, and among over 300 serum biomarkers, we identified apolipoprotein A-I (APOA1) and chemokine (C-C motif) ligand 17 (CCL17) as being predictive for both immune response to IMA901 and overall survival. A randomized phase 3 study to determine the clinical benefit of treatment with IMA901 is ongoing.

Therapeutic cancer vaccines hold the promise of combining meaningful efficacy (prolongation of survival) with very good safety and tolerability, as has been shown in several recent randomized trials<sup>1–3</sup>. However, development of cancer vaccines remains a major challenge, with little knowledge of (i) the optimal tumor antigens to target, (ii) suitable agents to counteract regulatory mechanisms opposing successful immunotherapy and (iii) surrogate and predictive biomarkers that can improve our understanding of these regulatory mechanisms and predict a patient's response to therapy.

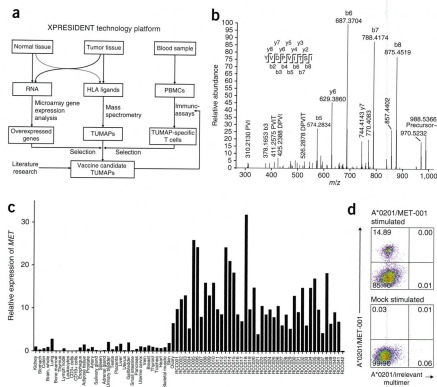
The first major issue addressed in this work is whether relevant HLA-restricted peptides for immunotherapeutic intervention in patients with

RCC can be identified and clinically validated. We defined the relevance of the antigens as their natural presence on the tumor in the majority of RCC samples, their immunogenicity (induction of T cell responses in clinical studies) and the association of the vaccine-induced T cell responses with clinical benefit. For the identification, selection and pre-clinical immunological validation of such antigens, we used the antigen discovery platform XPRESIDENT<sup>4,5</sup> to create a multipeptide vaccine designated IMA901 for immunotherapy of RCC. We tested IMA901 in HLA-A\*02+ subjects with advanced RCC in two clinical trials, a phase 1 ( $n = 28$ ) and a randomized phase 2 ( $n = 68$ ) trial, both of which assessed the association of T cell responses to IMA901 with clinical benefit.

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**Figure 1** Key components of the XPRESIDENT technology platform to identify and select naturally presented TUMAPs. (a) Process scheme showing the steps from primary tumor tissues and blood samples to a multipetide immunotherapeutic cancer vaccine. (b) Shown as an example is the sequencing of the peptide YDVPVITSI (MET-001) encoded by *MET* and naturally presented by HLA-A\*02 on primary RCC tissue by mass spectrometry. *m/z*, mass to charge ratio; b2–b8, b-series fragment ions; y2–y8, y-series fragment ions. (c) mRNA expression profile of *MET*. Expression values for bulk normal tissues and several RCC samples are shown relative to normal bulk kidney (with a value arbitrarily set to 1.0). Each bar represents a single microarray measurement. (d) HLA multimer analysis of *in vitro*-primed T cells generated by stimulation of human CD8<sup>+</sup> T cells derived from healthy donors with MET-001 (top) or an irrelevant peptide (bottom) using artificial antigen-presenting cells. The numbers in the quadrants indicate the percentage of CD8<sup>+</sup> cells.



The second aim was to identify an agent that reduced numbers of T<sub>reg</sub> cells and that improved the clinical efficacy of the vaccine. A single dose of cyclophosphamide has been used in several previous studies with the intention of inhibiting T<sub>reg</sub> cells<sup>6–9</sup>. However, a beneficial effect of single-dose cyclophosphamide had not previously been conclusively shown in a randomized clinical trial.

The third issue addressed was whether blood cell populations or serum factors could be identified as prognostic or predictive biomarkers. We investigated cell populations known to counteract T cell–based immunotherapy, such as T<sub>reg</sub> cells<sup>10</sup>, several populations of myeloid-derived suppressor cells (MDSCs)<sup>11,12</sup>, interleukin-10 (IL-10)-secreting and IL-17-secreting T cells<sup>13</sup> and dysfunctional T cell receptor  $\zeta$  (TCR- $\zeta$ )<sup>low</sup> or nitrotyrosine<sup>high</sup> T cells<sup>14,15</sup>, which might have an effect on the prognosis of patients with RCC. Lastly, we also screened >300 serum biomarkers for association with clinical outcome and immune response.

## RESULTS

### Identification of TUMAPs based on natural presentation

XPRESIDENT is an antigen discovery platform that allows for the identification of thousands of TUMAPs—collectively defined as the HLA ligandome or immunopeptidome—directly from primary tumor tissues (Fig. 1a). It uses a combination of mass spectrometry, gene expression profiling, literature-based functional assessment, *in vitro* human T cell assays and bioinformatics to select a set of candidate vaccine TUMAPs for the tumor type of interest<sup>4,16,17</sup>.

We systematically investigated 80 predominantly primary RCC tissues, including 32 evaluable HLA-A\*02<sup>+</sup> RCC samples, by mass spectrometry, resulting in the identification of peptides from housekeeping and tumor-associated antigens<sup>4,18</sup>. To identify potentially clinically relevant antigens, we compared the mRNA expression profiles of 42 RCC samples to those of 35 different healthy tissues<sup>19</sup> and tested the immunogenicity of candidate tumor-associated antigens determined by gene expression profiling through subsequent *in vitro* priming using artificial antigen-presenting cells<sup>16</sup> to detect antigen-specific T cells in peripheral blood cells of healthy donors. We selected

nine HLA-A\*02-restricted TUMAPs and one HLA-DR-restricted TUMAP that were derived from highly overexpressed tumor antigens and designated them IMA901 (Table 1). We added an HLA-A\*02-restricted peptide from hepatitis B virus (HBV) as a marker peptide for the phase 1 study<sup>20</sup> that was not part of the IMA901 formulation in further trials. Data from a representative TUMAP, MET-001, are shown in Figure 1b–d.

### Safety, immunogenicity and efficacy in the phase 1 study

To investigate safety and obtain preliminary results on the immunogenicity and potential clinical benefit of IMA901 in humans, we conducted an open-label, multicenter, single-arm phase 1 study in patients with advanced RCC. Subjects received up to eight IMA901 vaccinations, each preceded by administration of granulocyte-macrophage colony-stimulating factor (GM-CSF) as an immunomodulator (Fig. 2a). We enrolled 28 HLA-A\*02<sup>+</sup> subjects with RCC, 15 of whom had not received prior systemic treatment. The remaining 13 subjects had previously received up to three treatment lines, mostly consisting of cytokines, often in combination with chemotherapy. We observed no treatment-related serious adverse events or deaths during the study period. At a 3-month follow-up, disease had progressed in 16 subjects, 11 subjects had stable disease and 1 subject showed a partial response to therapy according to the Response Evaluation Criteria in Solid Tumors (RECIST).

Among 27 immune-evaluable subjects, 20 showed a vaccine-induced T cell response to at least one TUMAP and 8 responded to multiple TUMAPs. Fourteen subjects showed a response to the HBV-001 marker peptide. As detailed in the Online Methods, we analyzed responses after a single round of *in vitro* stimulation and culture to enable the detection of both low and high frequencies of vaccine-induced T cells in the peripheral blood of the subjects. The kinetics of the T cell responses determined at the time points of individual blood drawings before and throughout the course of the



**Table 1** Composition of the vaccine IMA901 and characteristics of the HBV peptide included in the phase 1 study

| Peptide                    | Antigen                           | HLA  | Overexpression | <i>In vitro</i> immunogenicity | Remarks on function and tumor relevance                                                                                                                                                                          | References |
|----------------------------|-----------------------------------|------|----------------|--------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| ADF-001 (SVASTITGV)        | PLIN2                             | A*02 | 6.0            | +                              | Major constituent of the surface of lipid droplets.                                                                                                                                                              | 4,49       |
| ADF-002 (VMAGDIYSV)        | APOL1                             | A*02 | 6.0            | +                              | Overexpressed in several cancers; established as a marker for RCC.                                                                                                                                               |            |
| AP0-001 (ALADGVQKV)        |                                   | A*02 | 7.0            | +                              | Secreted major apolipoprotein of high-density lipoprotein. Overexpression in RCC.                                                                                                                                | 4          |
| CCN-001 (LLGATCMFV)        | CCND1                             | A*02 | 3.0            | +                              | Cell cycle regulation. Overexpression and association with tumorigenesis and metastasis described for various tumors.                                                                                            | 4,50       |
| GUC-001 (SVFAGVGVG)        | GUCY1A3                           | A*02 | 2.2            | +                              | cGMP synthesis. Proangiogenic effects in tumors.                                                                                                                                                                 | 4          |
| K67-001 (ALFDGDPHL)        | PRUNE2                            | A*02 | 3.4            | +                              | Largely uncharacterized so far. Overexpression in RCC.                                                                                                                                                           | 4,18       |
| MET-001 (YVDPVITSI)        | MET                               | A*02 | 13.6           | +                              | Hepatocyte growth factor receptor tyrosine kinase, cell signaling. Various implications in malignant transformation and invasiveness of tumor cells.                                                             | 4,18,51    |
| MUC-001 (STAPPVHNV)        | MUC1                              | A*02 | 1.6            | +                              | Protection against pathogen binding to the cell surface; roles in cell signaling. Altered glycosylation patterns lead to new T cell epitopes in tumors.                                                          | 52–55      |
| RGS-001 (LAALPHSCL)        | RGS5                              | A*02 | 3.5            | +                              | Regulation of cell signaling. Overexpression during neovascularization in tumors.                                                                                                                                | 18,56      |
| MMP-001 (SQDDIKGIQKLYGKRS) | MMP7                              | DR   | 3.3            | +                              | Breakdown of extracellular matrix during tissue remodeling. Involved in tumor invasion and metastasis, tumor development and progression. Also, roles in apoptosis, cell proliferation and cell differentiation. | 57         |
| HBV-001 (FLPSDFPFVS)       | HBV; nucleocapsid protein (HbcAg) | A*02 | NA             | +                              | Marker peptide, not tumor associated. HbcAg is an antigenic determinant of HBV. Serological responses develop in most HBV-infected subjects, used for diagnosis of infection.                                    | 20,58      |

The characteristics of individual peptides contained in IMA901 are shown with peptide code (sequence), source antigen, HLA restriction, overexpression, *in vitro* immunogenicity, remarks on function and references. In the “overexpression” column, the ratios of mean expression in all analyzed RCC samples ( $n = 20$ ) compared with the mean expression in normal tissues are shown. In the column “*in vitro* immunogenicity,” “+” indicates that *in vitro* expansion of peptide-specific T cells was observed. NA, not applicable.

vaccinations were analyzed for 13 response courses in 9 subjects in detail and are shown for a representative subject in **Figure 2b**. We typically observed peak responses at 1–3 weeks after start of vaccinations, and we were still able to detect vaccine-induced immune responses 3 months after start of vaccinations in 7 of 13 response courses.

In a retrospective analysis, we found that subjects who responded to multiple TUMAPs were significantly ( $P = 0.019$ ) more likely to experience disease control (stable disease or partial response) than subjects who responded to only one TUMAP or had no response (**Fig. 2c**). In contrast, we found no association between T cell responses to HBV-001 and clinical benefit. To assess whether  $T_{reg}$  cells have a role in compromising immune responses in patients, we quantified the number of  $T_{reg}$  cells in the subjects before and after vaccination. Low percentages of  $T_{reg}$  cells before vaccination were associated with multiple T cell responses to the vaccine in comparison with nonresponders or single TUMAP immune responders (**Fig. 2d**;  $P = 0.016$ ).

### Safety and efficacy in the phase 2 study

We based the design of the phase 2 study on the observed positive association between disease control and T cell responses to multiple TUMAPs, as well as the negative association of  $T_{reg}$  cell numbers with the induction of such responses. We investigated whether an additional immunomodulator (single-dose cyclophosphamide) could improve the efficacy of IMA901 vaccination and the clinical outcome in patients with RCC, presumably by reducing the numbers of  $T_{reg}$  cells (**Fig. 3a**).

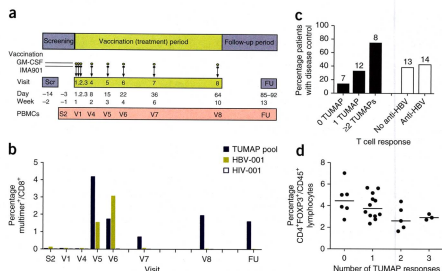
We randomized 1:1 a total of 68 HLA-A\*02+ subjects with metastatic RCC (intention-to-treat population, ITT) with documented progression during or after previous systemic therapy to receive either

single-dose cyclophosphamide before the first of 17 vaccinations or no pretreatment (33 subjects comprised the arm that included pretreatment with cyclophosphamide, termed here the “+Cy arm,” and 35 subjects comprised the arm with no pretreatment, termed here the “-Cy arm”); 64 subjects were evaluable for the predefined primary efficacy analysis (per protocol). The baseline characteristics and risk factors of the subjects were well balanced overall between the two randomization arms (**Supplementary Table 1**), except for a shorter median time since diagnosis in the subjects from the +Cy arm (which was reported to be a negative prognostic factor in previous studies<sup>21,22</sup>).

Confirming the phase 1 results, treatment with IMA901 was safe and generally well tolerated, with mild to moderate local site reactions being the most frequent side effects. Two possibly related serious adverse events were reported, with one subject experiencing a systemic allergic reaction after the twelfth vaccination (caused by GM-CSF, as shown by an *in vitro* basophil degranulation assay) and another with a grade 3 localized allergic reaction after the eleventh vaccination, with no similar signs of intolerance after further vaccinations. We found no differences in the safety profile between subjects in the +Cy arm and those in the -Cy arm.

Similar to reports for other cancer vaccines, shrinkage of established tumor lesions was infrequent, with one complete response and two partial responses being reported by investigators and one partial response as assessed by centralized review. The disease control rate (DCR; percentage of subjects with complete or partial response or stable disease according to RECIST within all treated subjects), according to a centralized review at 6 months after the start of treatment, was 31% (95% confidence interval (CI) 17–48%) in subjects with prior cytokine treatment and was 14% (95% CI 3–35%) in subjects

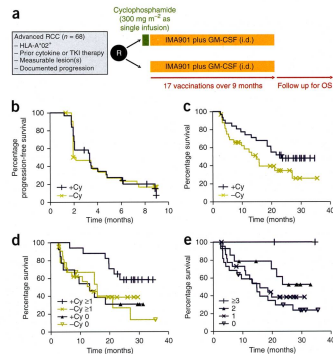
**Figure 2** Key observations of the IMA901 phase 1 trial. (a) Schedule of vaccinations with IMA901 plus the immunomodulator GM-CSF and of peripheral blood mononuclear cell (PBMC) sampling. Scr, screening; V, vaccination; FU, follow up (no vaccine applied). (b) The observed magnitude of T cell kinetics in a multimer analysis of a representative subject vaccinated with IMA901. The readout antigens were nine HLA class I TUMAPs in one sample (TUMAP pool), the marker peptide HBV-001, which was included in IMA901, and the negative control antigen HIV-001, which was not included in the vaccine. The timeline is shown in weeks, with each tick indicating 1 week. (c) DCR of subjects according to the presence or absence of detectable immune responses ( $n = 27$  subjects total). The numbers above the bars indicate number of subjects with the observed immune responses. Anti-HBV, vaccine-induced response to the HBV peptide. (d) Association of the number of T cell responses with the percentage of  $T_{reg}$  cells ( $n = 26$  subjects). Shown on the y axis is the percentage of CD4<sup>+</sup>FOXP3<sup>+</sup> cells among CD4<sup>+</sup> lymphocytes in prevaccination samples. Shown on the x axis is the number of vaccine-induced TUMAP responses per subject. Each dot represents an individual subject, and the horizontal lines represent the median values.



with prior tyrosine kinase inhibitor (TKI) treatment. Whereas progression-free survival (PFS) was comparable in the two study arms (Fig. 3b), we found a trend for prolonged survival in subjects in the +Cy arm (median overall survival of 23.5 months for +Cy compared with 14.8 months for -Cy; hazard ratio (HR) = 0.57,  $P = 0.090$ ; Fig. 3c and Supplementary Table 1).

### Association of immune responses and overall survival

Of the 64 per protocol subjects, 61 were evaluable for a prospectively defined immune response analysis. The immune response rate of 64% in this group (including 26% multi-TUMAP responders) was similar to that of the phase 1 study (where we investigated more time points).



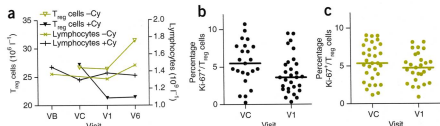
These rates were comparable between subjects with and without cyclophosphamide pretreatment (data not shown), indicating that cyclophosphamide did not alter the induction of T cell responses.

The baseline characteristics of the subjects were generally well balanced between the immune responders and the nonresponders and between multi-TUMAP responders and single-TUMAP responders or nonresponders (Supplementary Table 2). However, age was associated with fewer multi-TUMAP responses ( $P = 0.01$ ), and there was a tendency toward more responses in male than in female subjects. Neither age nor gender have otherwise been reported as prognostic factors for overall survival of patients with RCC<sup>22</sup>.

Despite the observed similarity of prognostic factors in the subjects in the +Cy and -Cy arms, a prospectively defined analysis showed that among immune responders, subjects had prolonged survival if pretreated with cyclophosphamide compared with subjects without this pretreatment (HR = 0.38,  $P = 0.040$ ) (Fig. 3d). In contrast, we found no difference in survival of subjects in the +Cy and -Cy arms among nonimmune responders (HR = 0.92,  $P = 0.870$ ). Although these data are based on small numbers of subjects because of the trial design, they suggest that a single dose of cyclophosphamide does not have antitumor activity by itself but instead supports the effects of the vaccine as an immunomodulator and enables the translation of immune responses into clinical benefit. Furthermore, survival time was extended if a subject had a response to multiple TUMAPs ( $P = 0.023$ ).

**Figure 3** Overall survival and PFS of subjects treated in the phase 2 trial. Evaluable subjects of the per protocol population are shown in the survival curves. (a) Design of the clinical trial. OS, overall survival; R, randomization; i.d., intradermal administration. (b) PFS of subjects treated with ( $n = 31$ ) or without ( $n = 33$ ) cyclophosphamide. (c) Overall survival of subjects treated with ( $n = 31$ ) or without ( $n = 33$ ) cyclophosphamide. (d) Overall survival of subjects evaluable for immune responses grouped as follows: immune responders, +Cy arm ( $n = 17$ ); subjects without an observed immune response, +Cy arm ( $n = 13$ ); immune responders, -Cy arm ( $n = 22$ ); subjects without an observed immune response, -Cy arm ( $n = 9$ ). (e) Overall survival of subjects with no detectable immune responses ( $n = 22$ ), immune responses to one TUMAP ( $n = 23$ ), immune responses to two TUMAPs ( $n = 14$ ) or immune responses to at least three TUMAPs ( $n = 2$ ).

**Figure 4** Modulation of  $T_{reg}$  cells by single-dose cyclophosphamide. (a) The medians of the absolute  $T_{reg}$  cell and lymphocyte counts of evaluable phase 2 subjects at different visits: VB, screening up to 4 weeks before V1; VC, administration of single-dose cyclophosphamide 3 d before V1; V1, first IMA901 vaccination; V6, sixth vaccination 3 weeks after V1.  $T_{reg}$  cells of evaluable ITT subjects pretreated with cyclophosphamide ( $n = 25$  at VC,  $n = 29$  at V1,  $n = 28$  at V6),  $T_{reg}$  cells of subjects not pretreated with cyclophosphamide ( $n = 31$  at VC,  $n = 27$  at V1,  $n = 27$  at V6), lymphocytes of subjects pretreated with cyclophosphamide ( $n = 35$  at VB,  $n = 35$  at V1,  $n = 34$  at V6) are shown. The decrease in the numbers of  $T_{reg}$  cells from VC to V1 was significant in the +Cy arm ( $P = 0.013$  by paired two-sided Wilcoxon test,  $n = 24$ ). (b,c) Phenotype of circulating  $T_{reg}$  cells in evaluable subjects treated with cyclophosphamide (b) ( $n = 23$  at VC,  $n = 29$  at V1) or not treated with cyclophosphamide (c) ( $n = 31$  at VC,  $n = 27$  at V1). Frequency of Ki-67<sup>+</sup> cells among the  $T_{reg}$  cells was significantly decreased from VC to V1 in subjects treated with cyclophosphamide ( $P = 0.0006$  by paired two-sided Wilcoxon test,  $n = 23$ ). Each dot represents an individual subject, and the horizontal lines represent the median values.



log-rank test for trend in subjects with increasing number of responses) (Fig. 3e), corroborating the observation from the phase 1 study that clinical benefit is associated with the breadth of vaccine-induced immune responses.

### Downmodulation of $T_{reg}$ cells by cyclophosphamide

To assess the effects of single-dose cyclophosphamide on the numbers of  $T_{reg}$  cells in patients with RCC, we characterized these cells<sup>23,24</sup> as CD45<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>FOXP3<sup>+</sup>CD25<sup>hi</sup>CD127<sup>low</sup> by immunophenotyping. A prospectively defined analysis showed an approximately 20% reduction in  $T_{reg}$  cell numbers 3 d after compared with immediately before the cyclophosphamide infusion in the +Cy arm ( $P = 0.013$ ); we found no such effect in the -Cy arm and no effect on absolute lymphocyte counts in subjects from either arm (Fig. 4a).

Furthermore, quantifying the expression of Ki-67 showed that the percentage of proliferating cells among all  $T_{reg}$  cells was decreased 3 d after compared with before cyclophosphamide treatment (Fig. 4b,c).

### Assessment of cellular biomarkers before treatment

To establish a profile of the immune-regulatory environment of patients with RCC, we analyzed different cellular biomarkers in samples from subjects of the phase 2 study obtained before treatment with cyclophosphamide and IMA901 and from matched healthy controls. MDSCs are myeloid cells with immunosuppressive properties that have been proposed to negatively modulate anticancer immunity. We developed a panel of antibodies to identify six MDSC phenotypes in a single multicolor staining: MDSC1 (CD14<sup>+</sup>, interleukin-4 receptor  $\alpha$  (IL-4R $\alpha$ )<sup>+</sup>), MDSC2 (CD15<sup>+</sup>IL-4R $\alpha$ )<sup>+</sup>, MDSC3 (Lineage<sup>+</sup>HLA-DR<sup>+</sup>CD33<sup>+</sup>)<sup>26,27</sup>, MDSC4 (CD14<sup>+</sup>HLA-DR<sup>+</sup>)<sup>28</sup>, MDSC5 (CD11b<sup>+</sup>CD14<sup>+</sup>CD15<sup>+</sup>)<sup>14</sup> and MDSC6 (CD15<sup>+</sup>FSC<sup>lo</sup>SSC<sup>hi</sup>)<sup>29</sup>. We found that the percentage of MDSC2–MDSC6 phenotypes among all lymphocytes were significantly ( $P < 0.01$ ) higher in subjects with RCC than in the controls (Fig. 5a and Supplementary Fig. 1a–e).

Two key mechanisms by which MDSCs cause T cell dysfunction have previously been reported: depletion of arginine, which induces T cell receptor  $\zeta$  chain downregulation<sup>14</sup>, and the generation of reactive oxygen species, which induces T cell tyrosine nitration<sup>30,31</sup>. Indeed, we found that TCR- $\zeta$  expression and nitrotyrosine expression by T cells—both measured as median fluorescence—were significantly lower ( $P < 0.0001$ ) and higher ( $P = 0.0038$ ), respectively, in patients compared with controls (Fig. 5b,c). TCR- $\zeta$  expression was significantly inversely correlated with numbers of MDSC2–MDSC4 ( $P < 0.05$ , data not shown) and strongly inversely correlated with

MDSC5 ( $P < 0.0001$ ; data not shown). We found no association between nitrotyrosine expression and the numbers of phenotypes MDSC1–MDSC6 (data not shown).

IL-17<sup>+</sup>CD4<sup>+</sup> T cells (T helper type 17 (T<sub>H</sub>17) cells) represent a distinct lineage of helper T cells with proinflammatory effector functions. T<sub>H</sub>17 cells have been found to be elevated in several human cancers<sup>13,32,33</sup> and may predict metastatic progression<sup>13</sup>. Comparison of patients with RCC and controls (Fig. 5d,e and Supplementary Fig. 1f,g) showed that T<sub>H</sub>17 cells were significantly enriched in the peripheral blood of subjects with RCC ( $P < 0.0001$ ; Fig. 5d). Furthermore, the concentrations of IL-10<sup>+</sup>CD4<sup>+</sup> T cells were also significantly higher in patients with RCC than in controls ( $P < 0.0001$ , Fig. 5e).

In a retrospective analysis, the numbers of two out of the six MDSC phenotypes were significantly negatively associated with overall survival: MDSC4 ( $P < 0.001$  by continuous analysis; patients with high compared with low numbers of MDSC4 are shown in Fig. 5f) and MDSC5 ( $P = 0.016$ ). TCR- $\zeta$  expression tended to associate positively with survival ( $P = 0.084$ ).

### Assessment of serum biomarkers before treatment

To identify serum parameters that could predict a potentially increased immunogenicity and overall survival by IMA901 treatment, we measured >300 analytes in samples from subjects in the IMA901 phase 2 study before treatment. To distinguish prognostic biomarkers (that is, those associated with clinical outcome independent of therapy) from predictive biomarkers, we considered only those biomarkers that were associated with both immune response and with overall survival in the +Cy arm but not in the -Cy arm. The use of this method was justified by the observation that immune responses to TUMAPs were associated with clinical benefit in both clinical studies described here (whereas responses to HBV-001 were not) and by the finding that subjects from the +Cy arm showed longer overall survival times (HR = 0.57,  $P = 0.090$ ) and better association of immune response with overall survival compared with subjects in the -Cy arm.

Post-hoc univariate statistical analyses revealed that serum concentrations of two analytes, APOA1 and CCL17, were positively predictive for immune responses ( $P = 0.016$  and  $P = 0.032$ , respectively) and multipoint responses ( $P < 0.0001$  and  $P = 0.0028$ , respectively; data not shown). High concentrations of APOA1 and CCL17 were able to identify patient populations with significantly longer overall survival ( $P < 0.007$  and  $P < 0.011$ , respectively), with this effect being pronounced in the +Cy arm but absent in the -Cy arm (Fig. 5g–j).

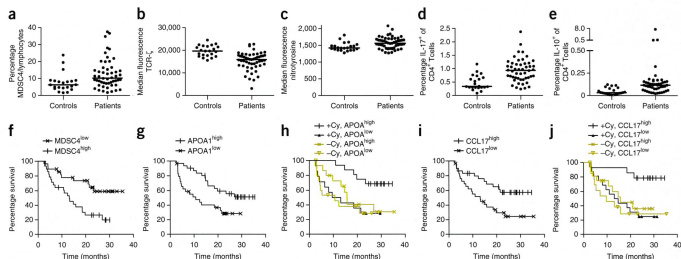


**Figure 5** Analysis of pretreatment biomarkers. (a–e) Comparison of cellular biomarkers of evaluable ITT subjects aged <70 years ( $n = 50–53$  per group) and age- and gender-matched healthy controls ( $n = 22$ ). Each dot represents an individual subject, and horizontal lines represent the median values. Statistical assessments were performed using unpaired two-sided Wilcoxon tests. MDSC4,  $P = 0.0043$  (a); TCR- $\gamma$ ,  $P < 0.0001$  (b); nitrotyrosine expression of T cells,  $P = 0.0038$  (c); IL-17 $^{+}$ ,  $P < 0.0001$  (d); IL-10 $^{+}$ ,  $P < 0.0001$  (e). (f) Overall survival of evaluable per protocol subjects having high ( $n = 29$ ) or low ( $n = 28$ ) MDSC4 numbers; HR = 0.35,  $P = 0.0035$  by log-rank test. (g–j) Overall survival in evaluable per protocol subjects having high or low concentrations of APOA1 (g) or CCL17 (i); subgroup analyses were performed with respect to cyclophosphamide pretreatment (h,j). The HR for overall survival in APOA1 $^{high}$  compared with APOA1 $^{low}$  subjects was 0.41 ( $P = 0.007$  by log-rank test,  $n = 31$  compared with  $n = 30$ , respectively), 0.26 in the +Cy arm ( $P = 0.006$ ,  $n = 16$  compared with  $n = 14$ ) and 0.62 in the –Cy arm ( $P = 0.278$ ,  $n = 15$  compared with  $n = 16$ ). The HR for overall survival in CCL17 $^{high}$  compared with CCL17 $^{low}$  subjects was 0.41 ( $P = 0.011$  by log-rank test,  $n = 30$  compared with  $n = 30$ , respectively), 0.19 in the +Cy arm ( $P = 0.003$ ,  $n = 14$  compared with  $n = 16$ ) and 0.69 in the –Cy arm ( $P = 0.418$ ,  $n = 16$  compared with  $n = 14$ ).

## DISCUSSION

To the best of our knowledge, this work is the first to show that HLA-restricted peptides, selected by virtue of being naturally presented by cancer, elicit specific vaccine-induced immune responses that are associated with clinical benefit. Most therapeutic vaccine trials in cancer so far have not shown such an association, which may be explained by the lack of confirmation of natural presentation on patient-derived primary tumor tissue. In two trials testing IMA901, the breadth of the T cell response (that is, the number of different specific antigens against which T cell responses were induced by the vaccine) was significantly associated with clinical benefit, implying that the targeting of multiple antigens in immunotherapy is required for clinical efficacy. The finding that immune responses are associated with survival only in subjects randomized to a single dose of cyclophosphamide; and (iii) the baseline characteristics were overall well matched between multipetite immune responders, immune responders and nonresponders (Supplementary Table 2), and the two observed differences (age and sex) are not considered prognostic factors in advanced RCC.

Moreover, this is the first randomized trial to show that single-dose cyclophosphamide is associated with prolonged survival after tumor immunotherapy. This effect is only observed in subjects with specific immune responses to IMA901, indicating that single-dose cyclophosphamide does not have any single-agent activity (either as a cytotoxic or as a nonspecific immunomodulatory drug) but rather acts as an

immunomodulator of the vaccine. Differences in baseline factors cannot explain this better outcome; if anything, subjects pretreated with cyclophosphamide tended to have a slightly worse prognosis, as exemplified by the shorter time from tumor diagnosis to randomization in this group<sup>21,22</sup>. The results also show that cyclophosphamide predominantly affects proliferating  $T_{reg}$  cells within the  $T_{reg}$  population. A prior report suggested that peripheral  $T_{reg}$  cells have a higher fraction of proliferating cells than do other cell subsets<sup>34</sup>.

The survival of patients who received cytokine treatment prior to IMA901 vaccination plus cyclophosphamide (with the median overall survival not reached after 33.1 months) compared favorably with data recently published from studies with TKIs in the same setting (median overall survival of 17.8 months for sorafenib treatment compared with 15.2 months for placebo<sup>35</sup> and 16.4 months for sunitinib treatment<sup>36</sup>), whereas the overall survival of patients receiving IMA901 vaccination without cyclophosphamide (median overall survival of 15.8 months) matched these trials quite well. The baseline characteristics and risk factors of the subjects in the IMA901 phase 2 trial were generally comparable in this inter-trial comparison (Supplementary Table 3). Although encouraging, this observation must be interpreted with caution, as it was derived from a comparison with historical controls.

Our work also suggests that, in addition to  $T_{reg}$  cells, MDSCs may have a role in RCC outcome. Among several MDSC phenotypes described in the literature, only two populations, here termed MDSC4 and MDSC5, were shown to be negatively associated with survival in patients with RCC. Similar cellular populations have been shown to be prognostic in breast and colon cancer<sup>37</sup>. This observation is new for RCC and for cancer under immunotherapy. The TKI sunitinib, a standard first-line treatment in advanced RCC, has been shown to decrease the numbers of  $T_{reg}$  cells in mice<sup>38</sup> and patients with RCC<sup>39</sup>, as well as MDSCs in patients with RCC<sup>40</sup>. Based on this

rationale, a randomized phase 3 trial combining IMA901 with single-dose cyclophosphamide and sunitinib compared with treatment with sunitinib alone was started recently<sup>41</sup>.

The retrospective screening of a large number of serum biomarkers and their association with several outcome variables showed that two biomarkers, APOA1 and CCL17, are potentially predictive for vaccine-induced immunity and overall survival. APOA1 is the major protein component of high-density lipoprotein. Reduced concentrations of high-density lipoprotein and APOA1 are found in several types of cancer<sup>42</sup> and are associated with chronic inflammatory and oxidative stress, which may suppress adaptive immune responses<sup>43</sup>. CCL17 is a chemottractant for several immune cells, including CD4<sup>+</sup> and CD8<sup>+</sup> T cells, in particular, skin-homing<sup>44,45</sup> and tumor-infiltrating T cells<sup>46</sup>. A major source of CCL17 is myeloid dendritic cells; constitutively as well as induced production of CCL17 has a role in dendritic cell function<sup>47,48</sup>. Based on these exploratory biomarker results, we hypothesize that high serum concentrations of APOA1 and CCL17 may predict immune and clinical response to IMA901.

Several aspects of the study reported here strongly suggest that therapeutic vaccinations with IMA901 induce specific immune responses that lead to clinical benefit and may be predicted by a defined pretreatment biomarker pattern. However, relatively small numbers of patients have been evaluated thus far, requiring further confirmation of clinical benefit as a prospectively defined endpoint in a large randomized clinical trial, which is currently ongoing. This randomized trial will also serve to validate the proposed predictive biomarker signature in a prospectively defined fashion.

Finally, we conclude that the rational clinical development of therapeutic cancer vaccines accompanied and guided by a comprehensive biomarker program—including T cell response monitoring, as well as monitoring of other cellular and serum biomarkers—is feasible even in multicenter trials and is valuable, provided that sample quality is tightly controlled. Such biomarkers reflect relevant mechanisms of immune induction and tolerance and assist in defining patient subpopulations with a higher prospect of response to cancer immunotherapy. These findings may have relevance for other cancer immunotherapy trials.

## METHODS

Methods and any associated references are available in the online version of the paper.

*Note: Supplementary information is available in the online version of the paper.*

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## AUTHOR CONTRIBUTIONS

S.W. designed and supervised the immunomonitoring and cellular biomarker program. T.W. designed and supervised the antigen discovery and the noncellular biomarker program. T.W. and H.S.-J. designed the composition of the drug.

A.S. was the principal investigator of the IMA901 phase 1 and 2 studies and participated in the discovery phase. A.S., M.S. and P.-Y.D. treated a substantial number of patients in the phase 1 study; R.Z., A.P. and C.S. treated a substantial number of patients in the phase 2 study; W.B. was involved in the discovery phase and immunomonitoring and recruited patients in the phase 2 study; R.M., N.H. and D.M. performed and contributed essentially to the immunomonitoring. R.M. and N.H. performed and contributed essentially to the cellular biomarker program. N.H. and O.S. contributed essentially to the discovery phase and design of the clinical trials. J. Fritsche performed the bioinformatics analyses. A.M. and J. Fritsche performed and contributed essentially to the noncellular biomarker program. V.V. and C.T. supervised and performed experiments in the discovery phase. P.L. and C.F. supervised and performed the pharmaceutical development, respectively. H.P. and J.J.S. contributed essentially to the PBMC preparations for immunomonitoring. V.B., S.M., G.P. and E.D. were essentially involved in the design and data analysis of the cellular biomarker program. E.D. and T.B. performed some of the cellular biomarker experiments. H.Y., T.M., F.H., N.T., K.H. and H.T. were involved in the discovery phase. H.-G.R. and S.S. contributed essentially to the discovery phase and to the design of the overall treatment approach. H.-G.R. contributed essentially to the design of the clinical phase 1 and 2 studies. A.M.-M., H.S.-J. and J. Frisch designed the phase 1 study and planned the analysis. A.M.-M. and J. Frisch supervised the conduct and the analysis of the phase 1 study. A.K., J. Frisch and H.S.-J. designed the phase 2 study and planned the analysis. A.K. and J. Frisch supervised the conduct of the phase 2 study. A.K. and C.R. supervised the analysis of the phase 2 study. S.W., H.S.-J., T.W. and C.R. wrote the manuscript. G.P., V.B. and H.-G.R. provided critical input in the writing of the manuscript. J. Frisch and C.R. had the overall medical oversight. H.S.-J. had the overall scientific oversight. H.-G.R. provided the conceptual basis for the work presented here.

## COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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# Lineage tracing and genetic ablation of ADAM12<sup>+</sup> perivascular cells identify a major source of profibrotic cells during acute tissue injury

Sophie Dulauroy<sup>1,2</sup>, Selene E Di Carlo<sup>1,2</sup>, Francina Langa<sup>3</sup>, Gérard Eberl<sup>1,2</sup> & Lucie Peduto<sup>1,2</sup>

Profibrotic cells that develop upon injury generate permanent scar tissue and impair organ recovery, though their origin and fate are unclear. Here we show that transient expression of ADAM12 (a disintegrin and metalloprotease 12) identifies a distinct proinflammatory subset of platelet-derived growth factor receptor- $\alpha$ -positive stromal cells that are activated upon acute injury in the muscle and dermis. By inducible genetic fate mapping, we demonstrate *in vivo* that injury-induced ADAM12<sup>+</sup> cells are specific progenitors of a major fraction of collagen-overproducing cells generated during scarring, which are progressively eliminated during healing. Genetic ablation of ADAM12<sup>+</sup> cells, or knockdown of ADAM12, is sufficient to limit generation of profibrotic cells and interstitial collagen accumulation. ADAM12<sup>+</sup> cells induced upon injury are developmentally distinct from muscle and skin lineage cells and are derived from fetal ADAM12<sup>+</sup> cells programmed during vascular wall development. Thus, our data identify injury-activated profibrotic progenitors residing in the perivascular space that can be targeted through ADAM12 to limit tissue scarring.

Skeletal muscle and skin have exceptional regenerative capacity and can undergo many rounds of repair in response to injury<sup>1,2</sup>. Although parenchymal regeneration is ensured by tissue stem cells, efficient tissue repair requires activation of the surrounding stromal micro-environment, mainly  $\alpha$ -smooth muscle actin-positive ( $\alpha$ -SMA<sup>+</sup>) myofibroblasts, to produce growth and survival factors, proinflammatory chemokines and components of the extracellular matrix, such as collagens. Inappropriate activation of the stromal compartment, however, leads to persistence of  $\alpha$ -SMA<sup>+</sup> collagen-overproducing cells (also called profibrotic cells), extracellular matrix accumulation and overgrowth of fibrous or adipogenic tissue, thereby compromising organ recovery and impairing function<sup>3</sup>. In the skeletal muscle, overgrowth of fibrous tissue has been suggested to originate from muscle stem cells exposed to environmental modifications associated with aging or injury<sup>4,5</sup>. In addition, resident platelet-derived growth factor receptor- $\alpha$ -positive (PDGFR- $\alpha$ <sup>+</sup>) mesenchymal cells distinct from muscle stem cells proliferate early upon muscle damage to promote tissue regeneration and show fibrogenic-adipogenic bipotential *in vitro*<sup>6,7</sup>. Accordingly, chronic activation of PDGFR- $\alpha$  leads to widespread organ fibrosis in mice<sup>8</sup>.

Myofibroblasts are a functionally heterogeneous population of cells and, at least *in vitro*, can be generated from several cell types, including circulating bone marrow-derived CD45<sup>+</sup>CD34<sup>+</sup> fibrocytes, fibroblasts, epithelial cells, mesenchymal stem cells, perivascular cells and nerve-associated cells<sup>9–13</sup>. Genetic cell-fate mapping would allow the identification of relevant precursors to profibrotic cells *in vivo*, in the context of acute muscle and dermis injury, with minimal

perturbation of the biological system being investigated<sup>14</sup>. However, such genetic approaches require specific markers that have yet to be reported for profibrotic cells.

ADAM12 is a membrane-anchored metalloprotease that is implicated in the signaling of epidermal growth factor, insulin-like growth factor and transforming growth factor- $\beta$  (TGF- $\beta$ )<sup>15–17</sup>. ADAM12 is transiently expressed during embryonic morphogenesis of skeletal muscles and visceral organs<sup>18</sup> and is re-expressed in several human diseases with a fibrotic component, such as liver and muscle injuries, scleroderma and desmoplastic tumors including aggressive fibromatosis<sup>19–23</sup>. Such a restricted and specific expression pattern prompted us to investigate the identity and role of ADAM12<sup>+</sup> cells during injury. Using models of acute injury in the muscle and the dermis, we demonstrate here that ADAM12<sup>+</sup> cells are programmed during vascular wall development and are re-induced by injury as a distinct subset of PDGFR- $\alpha$ <sup>+</sup> perivascular progenitors with a specific profibrotic fate and function. Accordingly, such cells can be specifically targeted to limit the generation of profibrotic cells and collagen accumulation during scarring.

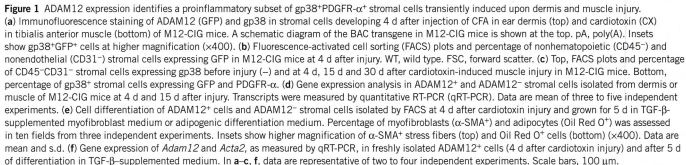
## RESULTS

### Injury induces the development of ADAM12<sup>+</sup> PDGFR- $\alpha$ <sup>+</sup> cells

To label ADAM12<sup>+</sup> cells, we generated transgenic mice, termed M12-CIG, expressing GFP and Cre recombinase under control of the *Adam12* locus on a bacterial artificial chromosome (BAC; **Fig. 1a**). We have shown earlier that acute tissue injury induced by complete Freund's adjuvant (CFA) injection into the ear dermis induces, within days, a massive generation of proinflammatory gp38<sup>+</sup> myofibroblasts

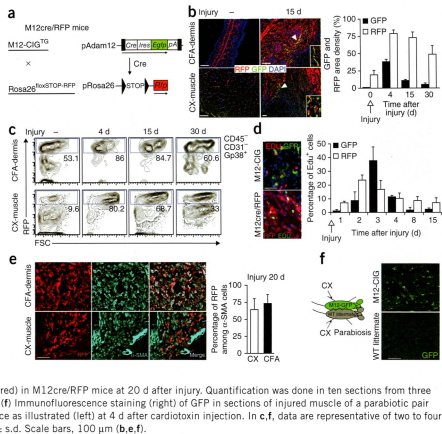
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The potential of injury-induced ADAM12<sup>+</sup> to differentiate into  $\alpha$ -SMA<sup>+</sup> myofibroblasts was confirmed *in vitro*. ADAM12<sup>+</sup> stromal cells isolated 4 d after injury were mostly spherical and poorly adherent. When grown in TGF- $\beta$ -supplemented medium, the majority of cells (>85%) differentiated into adherent  $\alpha$ -SMA<sup>+</sup> myofibroblasts with prominent stress fibers (Fig. 1e). However, in contrast to the case with ADAM12<sup>-</sup> cells, we observed only minor differentiation of ADAM12<sup>+</sup> cells into adipocytes when they were grown in adipocyte differentiation medium. Consistent with a transient progenitor state, ADAM12<sup>+</sup> cells

**Figure 2** The majority of myofibroblasts developing *in vivo* upon injury are progeny of ADAM12<sup>+</sup> cells. **(a)** Strategy for fate mapping of ADAM12<sup>+</sup> cells. **(b)** Immunofluorescence staining of ADAM12 (GFP, white arrowheads) and cellular progeny of ADAM12<sup>+</sup> cells (RFP) in M12cre/RFP mice before injury and 15 d after injection of CFA in ear dermis (top) or of cardiotoxin in tibialis anterior muscle (bottom). Proportion of GFP<sup>+</sup> cells (green) and RFP<sup>+</sup> cells (red) among total cells (blue) was measured by pixel quantification at the indicated time after injury. Values are mean and s.d. of CFA and cardiotoxin data;  $n = 5$ –10 sections from three independent experiments, with representative pictures. Insets show RFP<sup>+</sup>GFP<sup>+</sup> cells at higher magnification ( $\times 250$ ). **(c)** FACS analysis of stromal cells before injury (–) and at 4 d, 15 d and 30 d after CFA (top) or cardiotoxin (bottom) induced injury in M12cre/RFP mice. Numbers indicate the percentage of gp38<sup>+</sup> cells expressing RFP. **(d)** Proliferation rate of GFP<sup>+</sup> and GFP<sup>+</sup>RFP<sup>+</sup> cells in CFA-injured dermis of M12-CIG and M12cre/RFP mice, respectively ( $n = 10$  sections from two independent experiments). Representative pictures of Edu immunostaining in GFP<sup>+</sup> cells (3 d after injury) and in RFP<sup>+</sup> cells (15 d after injury) are shown on the left. DAPI (blue) stains the cell nuclei. Scale bar, 50  $\mu$ m. **(e)** Immunofluorescence staining of  $\alpha$ -SMA expression (cyan) by RFP<sup>+</sup> cells (red) in M12cre/RFP mice at 20 d after injury. Quantification was done in ten sections from three independent experiments (representative pictures). **(f)** Immunofluorescence staining (right) of GFP in sections of injured muscle of a parabiotic pair between M12-CIG mice and wild-type littermate mice as illustrated (left) at 4 d after cardiotoxin injection. In **c**, **d**, **e**, values are mean  $\pm$  s.d. Scale bars, 100  $\mu$ m (**b**, **e**, **f**).



downregulated expression of *Adam12* during differentiation to myofibroblasts while upregulating the transcript for *Acta2* (encoding  $\alpha$ -SMA; Fig. 1f). Together, these data indicate that a subset of gp38<sup>+</sup>PDGFR- $\alpha$ <sup>+</sup>Sca-1<sup>+</sup> cells, transiently expressing ADAM12 upon injury, preferentially differentiates into proinflammatory myofibroblasts.

### Injury-induced myofibroblasts are progeny of ADAM12<sup>+</sup> cells

ADAM12<sup>+</sup> cells were most prominent during the first week after injury and cell numbers declined thereafter (Fig. 1c). To determine the fate of ADAM12<sup>+</sup> cells *in vivo*, we mapped their cellular progeny by crossing M12-CIG mice to Cre-dependent *Rosa26<sup>loxSTOP-RFP</sup>* reporter mice<sup>30</sup> (M12cre/RFP, Fig. 2a). In such mice, all the progeny of ADAM12<sup>+</sup> cells was permanently labeled with RFP, whereas ADAM12<sup>+</sup> cells expressed both GFP and RFP. RFP<sup>+</sup> cells, present before injury albeit at low numbers, markedly expanded upon injury (Fig. 2b) to represent a major fraction of gp38<sup>+</sup> and PDGFR- $\alpha$ <sup>+</sup> stromal cells within a few days to 2 weeks after injury (Fig. 2c and Supplementary Fig. 2a, respectively) and then progressively decreased in number as the tissue healed. The expansion of RFP<sup>+</sup> cells followed extensive proliferation of ADAM12<sup>+</sup> cells in the first days after injury, whereas proliferation of RFP<sup>+</sup>GFP<sup>+</sup> cells was less marked but still substantial 15 d after injury (Fig. 2d). At 20 d after injury, 60–70% of  $\alpha$ -SMA<sup>+</sup> myofibroblasts were progeny of ADAM12<sup>+</sup> cells (Fig. 2e). In contrast, no muscle fibers or skin epidermal cells expressed RFP (Supplementary Fig. 2b), indicating that ADAM12<sup>+</sup> cells are developmentally distinct from muscle and skin tissue stem cells activated upon injury. Finally, a parabiosis experiment, in which an M12-CIG mouse and a wild-type partner were surgically sutured together, confirmed that ADAM12<sup>+</sup> cells were resident and not recruited from the bloodstream (Fig. 2f). Together,

these results indicate that a majority of gp38<sup>+</sup>, PDGFR- $\alpha$ <sup>+</sup> and  $\alpha$ -SMA<sup>+</sup> myofibroblasts generated *in vivo* upon muscle and dermis acute injury is derived from tissue-resident ADAM12<sup>+</sup> cells.

### ADAM12<sup>+</sup> profibrotic cells are induced *de novo* by injury

As ADAM12 is expressed during ontogeny<sup>18</sup>, and cells derived from ADAM12<sup>+</sup> progenitors were detected before injury (Fig. 2b, time 0), we investigated whether myofibroblasts were generated from ADAM12<sup>+</sup> cells induced *de novo* by injury. To that end, we carried out an inducible, tetracycline transactivator–based cell fate mapping experiment. We generated M12-TA/LC1/YFP mice that express tetracycline transactivator under control of the *Adam12* locus on a BAC (M12-TA<sup>Lox</sup>). Cre under control of the tetracycline transactivator (LC-1; ref. 31) and the conditional reporter *Rosa26<sup>loxSTOP-YFP</sup>* locus<sup>32</sup> (Fig. 3a). In these triple-transgenic mice, YFP labeling of the progeny of ADAM12<sup>+</sup> cells is controlled in time by the administration of doxycycline, which blocks the tetracycline transactivator–mediated labeling cascade, allowing for the distinct fate mapping of fetal and adult injury-induced ADAM12<sup>+</sup> cells (Fig. 3b).

First, we administered doxycycline continuously from conception until shortly before tissue injury (embryonic day 0 to postnatal day 23, E0–P23) to map the fate of adult ADAM12<sup>+</sup> cells induced *de novo* by injury. In that setting, and 10 d after injury, 50% (in the dermis) and 60% (in the tibialis anterior muscle) of the injured areas were infiltrated with YFP<sup>+</sup> cells (Fig. 3c, doxycycline E0–P23) with a CD45<sup>+</sup>gp38<sup>+</sup>PDGFR- $\alpha$ <sup>med/high</sup> phenotype (Fig. 3d), demonstrating that a major fraction of the injury-induced stroma is generated from ADAM12<sup>+</sup> cells induced *de novo* by injury. In these conditions, up to 53% of total gp38<sup>+</sup> cells in the whole tibialis anterior muscle were progeny of ADAM12<sup>+</sup> cells at 20 d after injury; this percentage

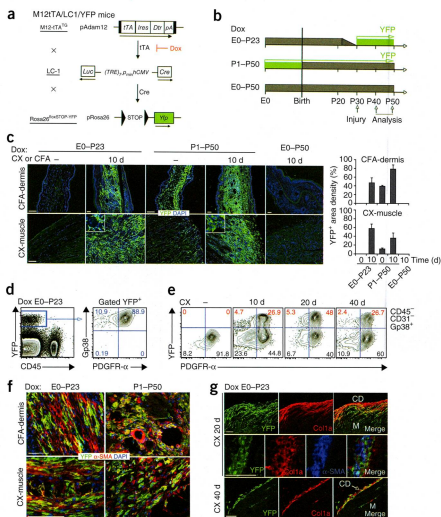
**Figure 3** ADAM12<sup>+</sup> profibrotic progenitors are induced *de novo* by tissue injury. **(a)** Strategy for inducible fate mapping and ablation of ADAM12<sup>+</sup> cells. tTA, tetracycline transactivator; Dtr, diphtheria toxin receptor; Luc, luciferase; TRE, tet-responsive element; hCMV, human cytomegalovirus; Dox, doxycycline.

**(b)** Experimental design for fate mapping of fetal and adult ADAM12<sup>+</sup> cells. E0, conception; P, postnatal day. **(c)** Immunofluorescence staining of YFP in ear dermis (top) and tibialis anterior muscle (bottom) of M12tTA/LC1/YFP mice treated as indicated. Quantification of YFP<sup>+</sup> cells (green) among total cells (blue) was assessed by pixel quantification in injured regions versus normal tissue. Values are mean  $\pm$  s.d.;  $n = 5$ –10 sections from three independent experiments, with representative images.

**(d)** FACS plots and percentage of YFP<sup>+</sup> cells expressing gp38 and PDGFR- $\alpha$  (right) in injured tibialis anterior muscles of M12tTA/LC1/YFP mice at 10 d after injecting cardiotoxin.

**(e)** FACS plots and percentage of total gp38<sup>+</sup> stromal cells expressing YFP and PDGFR- $\alpha$  in tibialis anterior muscle of M12tTA/LC1/YFP mice before injury, and at 10 d, 20 d and 40 d after injecting cardiotoxin. **(f)** Immunofluorescence analysis of  $\alpha$ -SMA expression (red) by fetal (right) and adult (left) progeny of ADAM12<sup>+</sup> cells (YFP) in M12tTA/LC1/YFP mice at 10 d after injury.

**(g)** Immunofluorescence staining of type I collagen (red) and  $\alpha$ -SMA (blue) by YFP<sup>+</sup> cells at indicated time after cardiotoxin-induced injury in M12tTA/LC1/YFP mice. M, muscle tissue; CD, collagen deposit. In **d**, **e**, **g**, we treated M12tTA/LC1/YFP mice with doxycycline from conception until 1 week before injury. In **d**, **g**, representative data from three to four independent experiments are shown. Nuclei are stained with DAPI. Scale bars, 100  $\mu$ m.



gradually decreased as the tissue healed (Fig. 3e). Most YFP<sup>+</sup> cells expressed  $\alpha$ -SMA<sup>low/med</sup>, consistent with progressive differentiation into  $\alpha$ -SMA<sup>+</sup> myofibroblasts (Fig. 3f). Furthermore, most progeny of injury-induced ADAM12<sup>+</sup> cells colocalized with interstitial collagen deposits, both during muscle regeneration and when healing was mostly achieved at 40 d after injury (Fig. 3g), showing that *de novo*-induced ADAM12<sup>+</sup> cells generated a subset of stromal cells targeted to fibrotic regions of the tissue.

The presence of a subset of  $\alpha$ -SMA<sup>+</sup>Col1A<sup>+</sup>YFP<sup>+</sup> cells (20–30% at 10 d after injury, Supplementary Fig. 3a) indicated that the efficiency of the tetracycline transactivator-based labeling system is not 100%, or that subsets of myofibroblasts arise from distinct origins and potentially carry different functions. At 40 d after injury, interstitial mesenchymal cells in regenerated normal muscle tissue were not progeny of ADAM12<sup>+</sup> cells (Supplementary Fig. 3b).

Next, we administered doxycycline continuously from birth (P1–P50) to map the fate of fetal ADAM12<sup>+</sup> cells. In the ear dermis, the progeny of fetal ADAM12<sup>+</sup> cells was already substantial before injury and further expanded to include most stromal cells after injury (Fig. 3c, P1–P50), indicating that ADAM12 is expressed during ontogeny by early mesenchymal progenitors (such as cranial neural crest cells, which generate all mesenchyme of the head<sup>33</sup>). In skeletal muscle, the progeny of fetal ADAM12<sup>+</sup> cells, which was limited before injury, expanded to 40% of injured areas (Fig. 3c, P1–P50).

In both ear dermis and muscle, the progeny of fetal ADAM12<sup>+</sup> cells included  $\alpha$ -SMA<sup>+</sup> cells (Fig. 3f) and other cell types (such as nerves, Supplementary Fig. 3c), in contrast to the progeny of *de novo*-induced ADAM12<sup>+</sup> cells. Thus, the progeny of fetal ADAM12<sup>+</sup> cells included both fibrogenic and nonfibrogenic lineages, but only cells of the fibrogenic lineage reexpressed ADAM12 upon acute injury (schematic diagram, Supplementary Fig. 4).

#### Ablation of injury-induced ADAM12<sup>+</sup> cells decreases fibrosis

To better define the role of ADAM12<sup>+</sup> cells and their cellular progeny in scarring, we sorted ADAM12<sup>+</sup>PDGFR- $\alpha$ <sup>+</sup>, ADAM12<sup>+</sup>PDGFR- $\alpha$ <sup>+</sup> and ADAM12<sup>+</sup>PDGFR- $\alpha$ <sup>+</sup> stromal cells from ADAM12-reporting mice (GFP<sup>+</sup> cells) at 4 d or 10 d after injury, as well as the progeny of ADAM12<sup>+</sup> cells (YFP<sup>+</sup> cells) from M12tTA/LC1/YFP mice at 20 d after injury. In the first weeks after injury, ADAM12<sup>+</sup> cells expressed *Col1a* transcripts more highly than ADAM12<sup>+</sup>PDGFR- $\alpha$ <sup>+</sup> cells, although the former had lower or similar expression of *Acta2* at 4 d and 10 d, respectively (Fig. 4a). At 20 d after injury, *Adam12* expression became undetectable in the progeny of ADAM12<sup>+</sup> cells, whereas the PDGFR- $\alpha$ <sup>med</sup> subset of this progeny most highly expressed *Col1a* and *Acta2*. These data demonstrate that ADAM12<sup>+</sup> cells and their cellular progeny are a subset of PDGFR- $\alpha$ <sup>high/med</sup> cells that produce excess amounts of type I collagen during differentiation toward the myofibroblast phenotype.



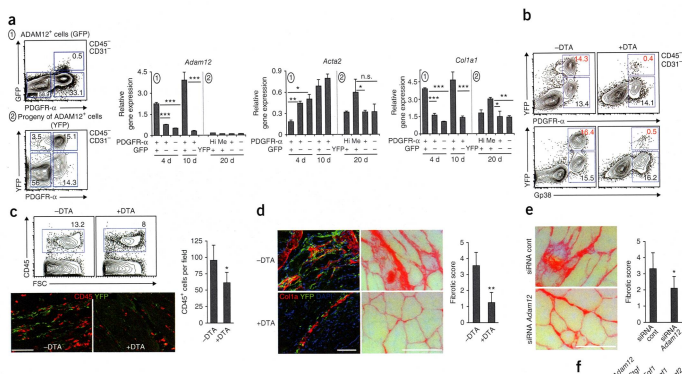


**Figure 4** Ablation of ADAM12<sup>+</sup> cells induced *de novo* by injury reduces inflammation and tissue fibrosis. **(a)** Expression of *Adam12*, *Acta2* and *Col1a1*, as measured by qRT-PCR, in the indicated cell populations isolated by FACS (gating strategy is shown on the left; numbers indicate the percentage of cells per gated type of cells in one representative experiment) from tibialis anterior muscle of m12-CIG mice (1) or M12TA/LC1/YFP mice (2) at the indicated times after cardiotoxin injection. Hi, high; Me, medium. Data are mean of two to four independent experiments. **(b)** FACS plots and percentage of CD45<sup>+</sup>CD31<sup>+</sup> stromal cells expressing YFP and PDGFR- $\alpha$  (top) or Gp38 (bottom) in injured tibialis anterior muscle of M12TA/LC1/YFP mice, 20 d after cardiotoxin injection, that were injected with DTA (right) or vehicle (PBS, left;  $n = 3$  independent experiments). **(c)** FACS plots and percentage of CD45<sup>+</sup> cells (top) and immunofluorescence staining of CD45 and YFP (bottom) in muscles treated as in **b**. Number of CD45<sup>+</sup> cells per injured area was counted in 10–15 sections from three independent experiments. **(d)** Immunofluorescence staining of type I collagen and YFP (left) and picrosirius staining (right) of sections from cardiotoxin-injured muscles treated as in **b**. **(e)** Picrosirius staining of cardiotoxin-injured tibialis anterior muscles of M12-CIG mice injected with siRNA targeting *Adam12*, compared with siRNA control. In **d**, **e**, we quantified fibrosis in picrosirius-stained sections at 3 weeks ( $n = 25$  fields from three to five independent experiments, representative images). Error bars, s.d.; \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0005$ , unpaired Student's *t*-test. **(f)** Expression of genes coding for growth factors were measured by qRT-PCR in cardiotoxin-injured muscles treated as in **e** ( $n = 4$  individual experiments, analysis of variance test). In **a–d**, M12TA/LC1/YFP mice were given doxycycline from conception until 1 week before injury. Scale bars, 100  $\mu$ m.

To formally demonstrate that ADAM12<sup>+</sup> cells and their progeny have a causal role in tissue fibrosis during acute injury, we injected M12TA/LC1/YFP mice, which also express the human diphtheria toxin receptor under control of the *Adam12* locus (Fig. 3a), with diphtheria toxin subunit A (DTA) to induce the ablation of ADAM12<sup>+</sup> cells. As we expected, injection of DTA induced a marked reduction of the progeny of ADAM12<sup>+</sup> cells (YFP<sup>+</sup> cells) (Fig. 4b) and of  $\alpha$ -SMA<sup>+</sup> stromal cells (Supplementary Fig. 5). Furthermore, consistent with the proinflammatory nature of ADAM12<sup>+</sup> cells (Fig. 1d), leucocyte infiltration decreased upon DTA treatment (Fig. 4c). Because of the loss of ADAM12<sup>+</sup> cells and their progeny, interstitial collagen was significantly reduced in injured tissues (Fig. 4d).

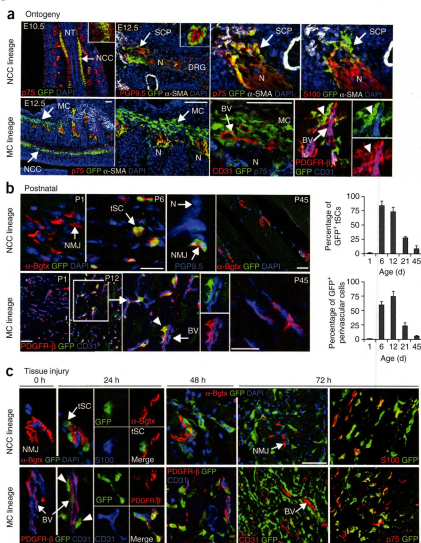
Next, to test whether ADAM12 itself has a role in fibrosis, we injected injured muscle of M12-CIG mice with siRNA directed against *Adam12*. At 3 weeks after injury, interstitial collagen within injured muscles was significantly reduced in treated mice (Fig. 4e), as was the expression of transcripts for stromal growth factors *Ctgf* and *Fgf1*. In contrast, growth factors essential for muscle regeneration such as *Igf1* and *Igf2* were increased when *Adam12* was silenced (Fig. 4f). As TGF- $\beta$  has a central role in wound healing and fibrosis

by stimulating expression of collagens<sup>34</sup>, we assessed whether ADAM12 might be involved in this process. Consistent with a role of ADAM12 in TGF- $\beta$  signaling, forced expression of *Adam12* in mouse fibroblastic cells *in vitro* increased *Col1a1* expression in response to TGF- $\beta$ , and silencing of endogenous *Adam12* decreased *Col1a1* expression upon TGF- $\beta$  stimulation (Supplementary Fig. 6a,b). Notably, *Adam12* itself is rapidly, although transiently, induced by TGF- $\beta$  *in vitro* (Supplementary Fig. 6c).

#### Injury reactivates ADAM12<sup>+</sup> cells in nerves and vascular wall

To clarify the identity of fetal and adult ADAM12<sup>+</sup> progenitors, we mapped ADAM12 expression from ontogeny until before injury. We detected ADAM12 expression in p75<sup>+</sup> neural crest cells (NCCs) at the outer border of the neural tube, starting at E9.5, when NCCs undergo an epithelial-to-mesenchymal transition and delaminate from the neural tube (Fig. 5a). NCCs subsequently migrate and give rise to multiple neural and non-neural cell types, including cells of the peripheral nervous system and craniofacial mesenchyme<sup>35</sup>. ADAM12 was downregulated in NCCs after delamination and re-expressed at E12.5 in NCC-derived p75<sup>+</sup> Schwann cell precursors (SCPs), as defined by the lack of detectable expression of the Schwann cell marker S100

**Figure 5** Tissue injury reactivates ADAM12<sup>+</sup> cells programmed during nerve and vascular wall development. (a) Immunofluorescence staining of ADAM12 (GFP) in sections from M12-CIG mouse embryos at the indicated embryonic age (days after conception), stained as indicated. White arrowheads, ADAM12<sup>+</sup>PDGFR- $\beta$ <sup>+</sup> perivascular cells in E12.5 embryos. Scale bars, 100  $\mu$ m. (b) Immunofluorescence staining of ADAM12 (GFP) in sections from tibialis anterior muscles of M12-CIG mice at the indicated age (postnatal days), stained as indicated. White arrowheads, ADAM12<sup>+</sup>PDGFR- $\beta$ <sup>+</sup> perivascular cells in muscles of 12-day-old pups. Scale bars, 50  $\mu$ m. (c) Immunofluorescence staining of ADAM12 (GFP) in sections from cardiotoxin-injured tibialis anterior muscle of M12-CIG adult mice at indicated time after cardiotoxin injection. White arrowheads, ADAM12<sup>+</sup>PDGFR- $\beta$ <sup>+</sup> perivascular cells. Similar results were obtained in CFA-injured dermis and cardiotoxin-injured skeletal muscle. Representative pictures of three independent experiments. Scale bar, 50  $\mu$ m. NCC, neural crest cells; MC, mesenchymal cells; NT, neural tube; DRG, dorsal root ganglia; N, nerve; SCP, Schwann cell precursor; BV, blood vessel; tSC, terminal Schwann cell; NMJ, neuromuscular junction.



calcium-binding protein- $\beta$  (S100) and close association with PGP9.5<sup>+</sup> axons<sup>36</sup> (Fig. 5a and Supplementary Fig. 7a). These findings are in accordance with earlier microarray data showing highest expression of ADAM12 in NCCs at E9.5 and in SCPs at E12.5 (ref. 37). From E10 to E14, ADAM12 was also expressed in p75<sup>+</sup> NCCs that migrated to the gut to generate the enteric nervous system (Supplementary Fig. 7a). In addition, at about E12.5, a population of ADAM12<sup>+</sup>PDGFR- $\beta$ <sup>+</sup>p75<sup>+</sup> $\alpha$ -SMA<sup>+</sup>CD31<sup>+</sup> mesenchymal cells was detected surrounding CD31<sup>+</sup> endothelium in close proximity to somites (Fig. 5a and Supplementary Fig. 7a). As PDGFR- $\beta$ -mediated signaling induces a pericyte fate in undifferentiated mesenchymal cells recruited to stabilize nascent vessels<sup>29</sup>, these data suggest that fetal ADAM12<sup>+</sup> mesenchymal cells include PDGFR- $\beta$ <sup>+</sup> pericyte progenitors. During postnatal growth (before weaning age), ADAM12 was transiently reexpressed in nonmyelinating S100<sup>+</sup> Schwann cells capping the axon terminals of the skeletal neuromuscular junction (NMJ), also called terminal Schwann cells (Fig. 5b), and in PDGFR- $\beta$ <sup>+</sup> perivascular cells, in both skeletal muscle and dermis (Fig. 5b and Supplementary Fig. 7b); the NMJ were visualized by staining with  $\alpha$ -bungarotoxin ( $\alpha$ -Bgtx), which binds the nicotinic acetylcholine receptors at the NMJ).

Together, these results show that ADAM12 is transiently expressed in distinct subsets of NCC-derived cells as well as in mesenchymal cells in close proximity to vessels, during nerve and vascular wall development respectively. Their progeny in adult muscle and dermis therefore includes nerves and glial cells on one hand (as described for NCCs<sup>35</sup>; Supplementary Fig. 3c) and mesenchymal perivascular cells on the other (Supplementary Fig. 8a), which might generate myofibroblasts upon injury through adult ADAM12<sup>+</sup> progenitor cells. We thus characterized adult ADAM12<sup>+</sup> cells induced

within hours of injury. Reminiscent of ADAM12 expression pattern during postnatal growth, ADAM12 was first reexpressed in S100<sup>+</sup> terminal Schwann cells capping the neuromuscular junctions and in PDGFR- $\beta$ <sup>+</sup> perivascular cells of injured regions (Fig. 5c). At 2–3 d after injury, ADAM12<sup>+</sup>p75<sup>+</sup>/S100<sup>+</sup> stromal cells not associated with the neuromuscular junction or blood vessels gradually infiltrated the interstitial stroma of injured regions (Fig. 5c). These results indicate that tissue injury reactivates ADAM12<sup>+</sup> cells programmed during nerve and vascular wall development.

### Perivascular ADAM12<sup>+</sup> cells are profibrotic progenitors

We found ADAM12<sup>+</sup> cells among NCC-derived Schwann cells and perivascular cells. As both cells of the NCC lineage and perivascular cells have the potential to generate myofibroblasts<sup>11,12,38</sup>, we mapped the fate of Wnt1<sup>+</sup> cells, as Wnt1 is expressed by NCCs<sup>39</sup> but not by perivascular cells. In Wnt1-Cre mice (expressing Cre under the control of the Wnt1 promoter) crossed to Rosa26<sup>loxSTOP-YFP</sup> mice, nerves and S100<sup>+</sup> Schwann cells (including terminal Schwann cells) in adult muscle and dermis were YFP<sup>+</sup>, and so were progeny of Wnt1<sup>+</sup> cells (Supplementary Fig. 8b). In addition, myofibroblasts developing upon ear injury expressed YFP, as mesenchyme of the head derives from NCCs<sup>35</sup> (Fig. 6a). However, in contrast to the progeny of ADAM12<sup>+</sup> cells (RFP<sup>+</sup> cells in Fig. 2b), no S100<sup>+</sup> stromal

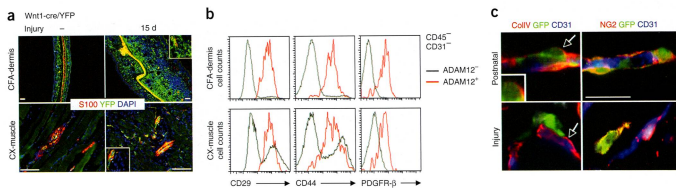


**Figure 6** Mesenchymal perivascular ADAM12<sup>+</sup> cells are profibrotic progenitors. **(a)** Immunofluorescence staining of YFP (progeny of Wnt1<sup>+</sup> cells, green) and S100 (Schwann cells, red) at 15 d after injection of CFA in ear dermis (top) and cardiotoxin in tibialis anterior muscle (bottom) in Wnt1-cre mice crossed to Rosa26<sup>loxSTOP-YFP</sup> mice. Nuclei are stained with DAPI (blue). Scale bars, 100  $\mu$ m. **(b)** Flow cytometric analysis of CD29, CD44 and PDGFR- $\beta$  expression by ADAM12<sup>+</sup> and ADAM12<sup>-</sup> cells isolated from CFA-injured ear dermis (top) and cardiotoxin-injured muscle (bottom) of M12-C1G mice at 72 h after injury. **(c)** Immunofluorescence staining of ADAM12 (GFP) in postnatal (P12) and cardiotoxin-injured (bottom, 24 h after injury) tibialis anterior muscle of M12-C1G mice. The basement membrane (collagen IV in red, white arrows) of microvessels (blue) is at left; pericyte staining with NG2-specific antibodies is at right. Nuclei are stained with DAPI (light gray). Representative pictures of three independent experiments. Scale bar, 25  $\mu$ m.

progeny of Wnt1<sup>+</sup> cells was found upon muscle injury (Fig. 6a), demonstrating that ADAM12<sup>+</sup> profibrotic progenitors developing in cardiotoxin-injured tibialis anterior muscles are not derived from the NCC lineage and ADAM12<sup>+</sup> Schwann cells, but rather from perivascular ADAM12<sup>+</sup> cells.

Consistent with this hypothesis, ADAM12<sup>+</sup> cells expressed PDGFR- $\beta$  and NG2, which are also expressed by pericytes (Fig. 6b,c), as well as CD29, CD44 (Fig. 6b), PDGFR- $\alpha$  and Sca-1 (Fig. 1c and Supplementary Fig. 1c), markers commonly used to identify multipotent mesenchymal progenitor cells in mice<sup>40,41</sup>. However, confirming our *in vitro* results (Fig. 1e), the progeny of ADAM12<sup>+</sup> cells induced *in vivo* upon injury was restricted to profibrotic cells and did not include other mesenchymal derivatives such as adipocytes, even in conditions favoring adipocyte differentiation *in vivo* (Supplementary Fig. 8c). ADAM12<sup>+</sup> cells induced upon injury were not embedded in the vascular basement membrane (the location of pericytes at steady state), in contrast to ADAM12<sup>+</sup> cells during postnatal growth (Fig. 6c). These data indicate that ADAM12 expression identifies a profibrotic subset of mesenchymal progenitors within the perivascular space.

## DISCUSSION

We found by inducible fate mapping *in vivo* that a majority of profibrotic cells developing upon acute muscle and dermis injury are generated from a distinct subset of PDGFR- $\alpha$ <sup>+</sup> proinflammatory stromal cells that are identified by transient expression of ADAM12 upon injury. Accordingly, genetic ablation of ADAM12<sup>+</sup> cells decreased tissue fibrosis during scarring. We further found that the profibrotic fate of ADAM12<sup>+</sup> cells is irreversible, as interstitial mesenchymal cells repopulating the healed tissue were not derived from ADAM12<sup>+</sup> cells. The progeny of ADAM12<sup>+</sup> cells was gradually eliminated as the tissue recovered, indicating that healing is associated with elimination of collagen-overproducing cells, rather than reversion to a normal, more quiescent mesenchymal phenotype. This is reminiscent of observations in the liver, in which resolution of fibrosis is associated with apoptosis of hepatic stellate cells, the primary collagen-producing cells in the liver<sup>42</sup>. Consistent with the proinflammatory nature of ADAM12<sup>+</sup> profibrotic progenitors, inflammation generally precedes and contributes to the development of fibrotic tissue, even though accumulation of fibrotic cells often outlasts inflammation<sup>3</sup>.

On the basis of *in vitro* differentiation assays, muscle stem cells have been suggested to have an alternative mesenchymal fate and generate fibrogenic cells<sup>4,5</sup>. Our *in vivo* lineage tracing data show that ADAM12<sup>+</sup> profibrotic progenitors do not contribute to parenchymal regeneration, indicating that they are distinct from muscle and skin stem cells. A detailed analysis shows that injury-induced ADAM12<sup>+</sup> cells include terminal Schwann cells capping the neuromuscular junction and PDGFR- $\beta$ <sup>+</sup> perivascular cells, two cell types endowed with high plasticity and roles in repair mechanisms of peripheral nerves and blood vessels, respectively<sup>29,43</sup>. We further demonstrate that ADAM12<sup>+</sup> cells of the mesenchymal lineage, but not of the Schwann cell lineage, are profibrotic progenitors in acute injured skeletal muscle. This finding is in agreement with recent reports showing that the perivascular niche of multiple organs contains pluripotent mesenchymal progenitors, which are suggested to be pericytes<sup>11,44</sup>. However, the cellular progeny of ADAM12<sup>+</sup> cells induced *de novo* by acute injury in the cardiotoxin and CFA models is restricted to profibrotic cells and does not include other mesenchymal derivatives such as adipocytes, suggesting that distinct subsets of mesenchymal progenitors, or progenitors at different stages of differentiation, are present in adult perivascular niches. In agreement with our findings, PDGFR- $\beta$  signaling opposes adipocyte differentiation in pericytes while activating immune genes<sup>45</sup>. In contrast to ADAM12<sup>+</sup> cells during postnatal growth, injury-induced ADAM12<sup>+</sup> cells are not embedded within the vascular basement membrane, suggesting that they are activated pericytes detaching upon injury or mesenchymal cells in close proximity to vessels. The expression of pericyte markers such as NG2 and PDGFR- $\beta$  would favor the first hypothesis. The detachment of pericytes from endothelium has been reported during vascular remodeling occurring in chronic pathologies, such as tumorigenesis, diabetic retinopathy and kidney disease<sup>46,47</sup>. These results are consistent with recent work in kidney fibrosis and spinal cord injury showing that subsets of pericytes are the major source of myofibroblasts and scar tissue<sup>48,49</sup>.

We further found that the Adam12 molecule has a role in tissue fibrosis. Notably, transcripts for growth factors with essential roles in muscle regeneration, such as *Igf1* and *Igf2*, were upregulated when *Adam12* was silenced *in vivo* during injury, suggesting that targeting *Adam12* alters stromal growth but favors tissue regeneration. These results are consistent with a report showing that transgenic

overexpression of *Adam12* under control of the muscle creatine kinase promoter both aggravates fibrosis and decreases muscle regeneration after injury, a phenotype further aggravated in the dystrophic background of mdx (X-linked muscular dystrophy) mice<sup>50</sup>.

ADAMs comprise several different protein modules<sup>51</sup>, including a catalytic domain, and disintegrin-like cysteine-rich domains which have been implicated in cell-cell interactions. Potential mechanisms underlying the profibrotic role of ADAM12 could therefore depend on the proteolytic release of membrane proteins (known substrates of ADAM12 include EGFR ligands<sup>15</sup>) or extracellular matrix proteins, or on cell-cell or cell-matrix interactions via its disintegrin domain. ADAM12 also enhances TGF- $\beta$ -induced transcriptional responses by modulating TGF- $\beta$  receptor trafficking<sup>17</sup>, consistent with our data showing that ADAM12 potentiates TGF- $\beta$ -mediated type I collagen synthesis in fibroblastic cells. As *Adam12* is rapidly, though transiently, induced by TGF- $\beta$  *in vitro* (our data and refs. 19,52), this suggests the hypothesis that ADAM12 has a role in the positive feedback loop of TGF- $\beta$  signaling. Nevertheless, further studies are required to determine whether this mechanism is relevant *in vivo* during injury. It also remains to be assessed whether ADAM12 has a direct role in the fate choice leading to the generation of  $\alpha$ -SMA<sup>+</sup> myofibroblasts rather than adipocytes.

Increasing evidence suggests that mesenchymal progenitors residing in adult organs have multiple developmental origins. Accordingly, an initial transient wave of mesenchymal stem cells in the postnatal bone derives from the neuroepithelial and neural crest cells<sup>53</sup>, whereas all mesenchymal cells of the head derive from the cranial neural crest<sup>54</sup>. Here we show that fetal ADAM12<sup>+</sup> cells programmed during vascular wall development contribute to the generation of mesenchymal perivascular cells in the adult skeletal muscle. We propose that reactivation of ADAM12<sup>+</sup> cells upon injury recapitulates an ontogenic program aimed at restoring vascular integrity, which, in the context of tissue damage and inflammation, leads to an inappropriate fibrotic outcome. The association of microvascular damage, inflammation and fibrosis characterizes several chronic human diseases including scleroderma (systemic sclerosis), a chronic connective tissue disease inducing extensive fibrosis of the skin and internal organs. Notably, ADAM12 is overexpressed in fibroblasts isolated from patients with scleroderma<sup>21</sup>. Future studies manipulating ADAM12<sup>+</sup> cells *in vivo* are needed to assess whether persistence of such cells in scleroderma and other chronic fibrotic diseases, such as liver fibrosis and aggressive fibromatosis<sup>19,21,22</sup>, has a causal role in the continuous generation of collagen-overproducing cells and fibrosis. Our work emphasizes that elimination versus persistence of functionally distinct subsets of mesenchymal cells might be key to restoring a healthy balance in extracellular matrix production during scarring, with implications for fibrotic diseases. As ADAM12<sup>+</sup> cells are rare in adult noninjured organs, we suggest that targeting injury-induced ADAM12<sup>+</sup> cells, or inhibiting ADAM12, might represent a new therapeutic approach to specifically limit the generation of collagen-overproducing cells.

## METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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## AUTHOR CONTRIBUTIONS

S.D. and S.E.D.C. carried out experiments and analyzed data. F.L. carried out BAC-mice transgenesis. G.E. participated in experiment design and data analysis, and wrote the manuscript. L.P. designed and directed the project, carried out experiments, analyzed data and wrote the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# GABAergic excitation after febrile seizures induces ectopic granule cells and adult epilepsy

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Temporal lobe epilepsy (TLE) is accompanied by an abnormal location of granule cells in the dentate gyrus. Using a rat model of complex febrile seizures, which are thought to be a precipitating insult of TLE later in life, we report that aberrant migration of neonatal-generated granule cells results in granule cell ectopia that persists into adulthood. Febrile seizures induced an upregulation of GABA<sub>A</sub> receptors (GABA<sub>A</sub>-Rs) in neonatally generated granule cells, and hyperactivation of excitatory GABA<sub>A</sub>-Rs caused a reversal in the direction of granule cell migration. This abnormal migration was prevented by RNAi-mediated knockdown of the Na<sup>+</sup>K<sup>+</sup>2Cl<sup>-</sup> co-transporter (NKCC1), which regulates the excitatory action of GABA. NKCC1 inhibition with bumetanide after febrile seizures rescued the granule cell ectopia, susceptibility to limbic seizures and development of epilepsy. Thus, this work identifies a previously unknown pathogenic role of excitatory GABA<sub>A</sub>-R signaling and highlights NKCC1 as a potential therapeutic target for preventing granule cell ectopia and the development of epilepsy after febrile seizures.

Disorders of neuronal migration during the development of the cerebral cortex result in neuronal heterotopia, which increases the risk of epilepsy<sup>1,2</sup>. Neuronal heterotopia is associated with spontaneous seizures and a reduced seizure threshold in many genetic<sup>3</sup>, drug<sup>4</sup> and lesion-induced<sup>5</sup> animal models, whereas rescue of the heterotopia decreases seizure susceptibility in a rat model of subcortical-band heterotopia<sup>6</sup>.

The abnormal location of hippocampal granule cells is also present in individuals with TLE and corresponding animal models<sup>7–11</sup>; the granule cell somata are dispersed, forming a wider granule cell layer (GCL) than normal (granule cell dispersion)<sup>7,8</sup>, whereas ectopically located granule cells are found in the dentate hilus (ectopic granule cells)<sup>10</sup>. Ectopic granule cells are abnormally incorporated into excitatory hippocampal networks<sup>10,12,13</sup> and may be epileptogenic. Here we focus on the mechanisms underlying the emergence and function of ectopic granule cells after experimental febrile seizures.

Febrile seizures are the most common convulsive events in humans between 6 months and 5 years of age, with a prevalence of 2–14%<sup>14</sup> in the population in this age range. Although febrile seizures are benign in most instances, 30–40% of them are 'complex'<sup>15,16</sup>, with a prolonged seizure duration of >15 min, and are subsequently associated with 30–70% of the cases of adult TLE<sup>17,18</sup>. Using a well-established rat model of complex febrile seizures<sup>19,20</sup>, which includes hippocampal hyperexcitability<sup>21,22</sup> and behavioral and electroencephalographic seizures in adulthood<sup>23</sup>, we sought a therapeutic strategy for preventing the emergence of epileptogenic foci.

## RESULTS

### Febrile seizures arrest newborn granule cells in the hilus

To determine whether febrile seizures affect the localization of neonatally generated granule cells, we placed rats at postnatal day 11 (P11 rats) under hyperthermic conditions at 39.5–42.5 °C (the hyperthermic group) (Fig. 1a). These rats exhibited a typical pattern of complex febrile seizures involving body flexion (Fig. 1b and Online Methods). Systemic seizures and epileptiform discharges in local field potentials (LFPs) were both prevented by pretreatment with pentobarbital, an activator of GABA<sub>A</sub>-R (Fig. 1c). Therefore, we used pentobarbital treatment in rats in this hyperthermic group as controls<sup>19</sup> in addition to another control group, a normothermic group not exposed to hyperthermic conditions.

To examine whether febrile seizures induce ectopic granule cells in adulthood, we labeled neonatally generated granule cells by injection of a retrovirus that encodes membrane-targeted GFP at P5 in rats from the hyperthermic and normothermic groups; we then immunostained sections from these rats with the granule cell marker prospero homeobox 1 (Prox1)<sup>24,25</sup> at P60 (Fig. 1a). At P60, we found GFP<sup>+</sup> granule cells in the GCL with extended well-oriented dendrites (Fig. 1d) in both the normothermic and hyperthermic groups ( $n = 6–8$  rats per group). In six of the eight rats in the hyperthermic group, however, we found ectopic granule cells, which had bipolar dendrites that extended into the hilus and axons that projected to the GCL, as well as into the CA3 region. We did not find these ectopic granule cells in the rats in the normothermic group ( $n = 0$  of 6 rats;  $P < 0.05$  for the normothermic compared to the hyperthermic group by Fisher's exact probability test) (Fig. 1e).

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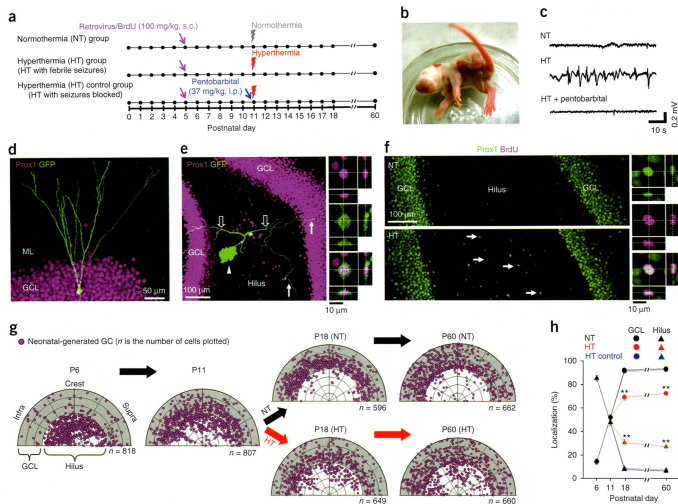


**Figure 1** Febrile seizures induce ectopic granule cells. **(a)** Experimental paradigms. Rats were killed at P6, P11, P18 or P60. s.c., subcutaneous; i.p., intraperitoneal. **(b)** Photograph of a P11 rat during febrile seizures. **(c)** LFP recording from the hippocampus of mice from each of the three experimental groups. **(d,e)** Retrovirus-infected GFP<sup>+</sup> normotopic **(d)** and ectopic **(e)** granule cells. Prox1<sup>+</sup> cells, presumably an aggregate of glial cells (arrowhead), were also found in regions near the retrovirally injected areas. The images on the far right are confocal three-dimensional reconstruction images of the soma. The arrows point to axons, and the open arrows point to dendrites. ML, molecular layer. **(f)** Images of dentate hilus from rats in the normothermic (NT) and hyperthermic (HT) groups at P60 immunolabeled for Prox1 and BrdU. Neonatally (P5)-generated granule cells (Prox1<sup>+</sup>BrdU<sup>+</sup>) in the hilus of a rat from the hyperthermic group are indicated by arrows. The images on the far right show a confocal three-dimensional reconstruction of a neonatally (P5)-generated granule cell. **(g,h)** Semicircular diagrams of the dentate gyrus **(g)** and a sequential line graph **(h)** showing the localization of neonatally (P5)-generated granule cells (GCs) as a function of age in postnatal days. Hippocampal sections were prepared at multiple postnatal time points (P6, P11, P18 and P60) and immunostained for Prox1 and BrdU **(g)**. The locations of neonatally (P5)-generated granule cells were analyzed and plotted on the diagrams (Online Methods). Localization (on the y axis, 100% corresponds to all neonatally (P5)-generated granule cells in the hilus and GCL of neonatally (P5)-generated granule cells in the hilus (triangles) and the GCL (circles)). \*\**P* < 0.01 compared to the normothermic group by Tukey's test after one-factor ANOVA (*n* = 9–16 sections from 3–4 rats).

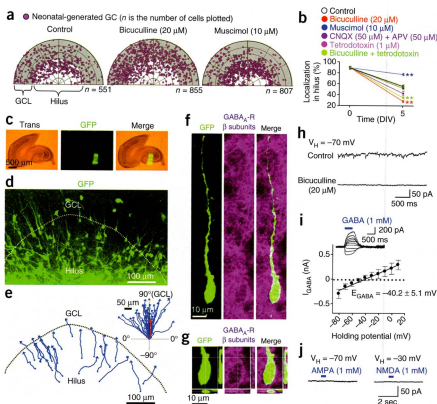
Although retroviral labeling enabled morphological analyses of ectopic granule cells, it was not suitable for quantitative analyses because the number of GFP-labeled neurons varied among the experiments. We thus labeled newly generated cells by a single-bolus injection of the S-phase marker 5-bromo-2'-deoxyuridine (BrdU) at P5. We killed the rats at either P6, P11, P18 or P60 for immunohistochemical staining with Prox1 (Fig. 1f). Most neonatally (P5)-generated, BrdU<sup>+</sup> granule cells were found in the hilus at P6 and moved to the GCL by P18 (Fig. 1g,h). The rats in the hyperthermic group had more BrdU<sup>+</sup> granule cells in the hilus than the rats in the normothermic group or the hyperthermic control group at both P18 and P60 (Fig. 1h), indicating that these ectopic granule cells were induced by seizures rather than hyperthermia. The ectopic granule cells appeared by P18 and

persisted into adulthood (P60) in the hyperthermic group. The densities of BrdU<sup>+</sup> granule cells in the hilus and the GCL, but not in the entire dentate gyrus, differed substantially between the normothermic, hyperthermic and hyperthermic control groups at P60 (Supplementary Fig. 1), implying that febrile seizures attenuated the migration, but not the proliferation or survival, of immature granule cells.

#### GABA<sub>A</sub>-R signaling regulates granule cell localization

GABA is crucial in neuronal migration during cortical development<sup>26–30</sup>. To examine whether GABA<sub>A</sub>-R signaling is involved in granule cell ectopia, we treated rat pups with a low concentration (1 mg per kg of body weight) of the GABA<sub>A</sub>-R antagonist picrotoxin once a day for 7 d after hyperthermic induction of febrile seizures (Supplementary Fig. 2a,b).

**Figure 2** GABA<sub>A</sub>-R signaling modulates granule cell migration. **(a, b)** Diagrams **(a)**; 5 DIV) and a sequential line graph **(b)** showing the locations of the neonatally (P5)-generated granule cells in cultured slices. The drugs indicated were applied from 0 DIV to 5 DIV.  $^{**}P < 0.01$  compared to control without reagents by Tukey's test after one-factor ANOVA ( $n = 6$ ). **(c)** Images of cocultures. Trans, transmitted light. **(d, e)** Images **(d)**; 4 DIV) and trajectories **(e)** of GFP<sup>+</sup> granule cells for 24 h (3–4 DIV) in the dentate gyrus of a coculture. The dotted lines in **d** and **e** indicate the boundary between the hilus and the GCL. The trajectories and orientation of 35 granule cells are shown **(e, right)**. **(f, g)** A GFP<sup>+</sup> granule cell (with the soma shown in **g**) immunostained for GABA<sub>A</sub>-R  $\beta$  subunits. **(h–j)** Response of GFP<sup>+</sup> cells in the hilus to GABAergic and glutamatergic inputs recorded under the gramicidin-perforated **(h, i)** or whole-cell **(j)** patch-clamp technique. Currents were recorded from GFP<sup>+</sup> cells **(h, j)** with or without the administration of drugs, and the current-voltage (*I*-*V*) relationships of the GABA-evoked currents are shown in **i**.  $V_H$ , holding membrane potential.



This subconvulsive dose of picrotoxin blocked seizure-induced granule cell ectopia. In contrast, repetitive administration of phenobarbital, a positive modulator of GABA<sub>A</sub>-R, increased the number of hilar granule cells in the rats in the hyperthermic group. In the rats in the normothermic group, phenobarbital increased the density of hilar ectopic granule cells but picrotoxin did not. Neither picrotoxin nor phenobarbital affected the total density of BrdU<sup>+</sup> cells in either group (Supplementary Fig. 2c). These results suggest that GABA<sub>A</sub>-R signaling regulates granule cell ectopia.

To confirm this phenomenon *ex vivo*, we prepared hippocampal slices from P6 rat pups and cultured them for 5 d in the presence of the GABA<sub>A</sub>-R antagonist bicuculline or the GABA<sub>A</sub>-R agonist muscimol. The granule cell localization pattern in these rats was similar to that observed *in vivo*: muscimol facilitated and bicuculline retarded granule cell migration (Fig. 2a, b). The sodium channel blocker tetrodotoxin did not affect granule cell localization or the effects of bicuculline treatment, which suggests that action-potential-independent, ambient GABA regulates granule cell migration. The receptors AMPA (AMPA-R) or NMDA (NMDA-R) were also probably not involved in this process (Fig. 2b).

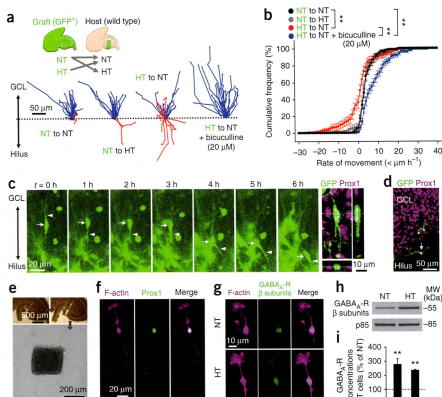
### Migrating granule cells receive excitatory GABA<sub>A</sub>-R inputs

Granule cell progenitors are associated with radial glia in the dentate gyrus<sup>31,32</sup>; however, direct time-lapse evidence for radially migrating granule cells is lacking. We visualized granule cell migration using a slice coculture system in which we replaced the hilar region of the hippocampal slices from wild-type rats with the hilar graft slices prepared from transgenic rats expressing GFP (GFP<sup>+</sup> transgenic rats) (Fig. 2c). We defined GFP<sup>+</sup> cells with single leading processes and trailing processes emanating from the oval-shaped somata whose minor axis was less than 10  $\mu$ m as immature granule cells because they expressed Prox1 but not the mature neuronal marker NeuN, the mature granule cell marker calbindin or the astrocyte markers S100 $\beta$  and glial fibrillary acidic protein (GFAP) (Supplementary Fig. 3a–c). After 5–12 d *in vitro* (DIV), most migrating granule cells reached the GCL and showed mature morphology (Supplementary Fig. 3a).

A 24-h time-lapse analysis revealed that granule cells migrated radially to the GCL (Fig. 2d, e, Supplementary Fig. 4a and Supplementary Video 1). The mean velocity and total distance of the cells were  $3.94 \pm 0.13$   $\mu$ m per h (mean  $\pm$  s.e.m.) and  $98.5 \pm 3.1$   $\mu$ m (mean  $\pm$  s.e.m.), respectively ( $n = 35$ ). The granule cells occasionally showed somal translocation to the GCL (Supplementary Fig. 4b and Supplementary Video 2). The leading processes of the granule cells attached to GFAP<sup>+</sup> radial glial scaffolds (Supplementary Fig. 4c). One of the 35 cells turned in the opposite direction in the middle of migration (Fig. 2e).

The migrating GFP<sup>+</sup> cells expressed GABA<sub>A</sub>-R  $\beta$  subunits on the membrane surface (Fig. 2f, g). To determine whether these GABA<sub>A</sub>-Rs were functional, we performed perforated whole-cell patch-clamp recordings by optically targeting two types of GFP<sup>+</sup> cells: cells in the hilus with the morphology of migrating granule cells (Fig. 2h–j) and cells in the GCL with the morphology of more mature granule cells (Supplementary Fig. 5). When clamped at  $-65$  mV, GFP<sup>+</sup> cells in the hilus had spontaneous inward currents that were abolished by a bath treatment with the GABA<sub>A</sub>-R antagonist bicuculline ( $n = 11$ ; Fig. 2h), whereas spontaneous inward currents in GFP<sup>+</sup> cells in the GCL were blocked with the simultaneous application of the non-NMDA-R antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and the NMDA-R antagonist 2-amino-5-phosphonovaleic acid (APV) ( $n = 11$ ; Supplementary Fig. 5a). Puff application of GABA evoked currents in all the GFP<sup>+</sup> cells tested: the reversal potential of the currents was  $-40.2 \pm 5.1$  mV (mean  $\pm$  s.e.m.) for GFP<sup>+</sup> cells in the hilus ( $n = 6$ ; Fig. 2i) and  $-64.8 \pm 6.3$  mV for GFP<sup>+</sup> cells in the GCL ( $n = 5$ ; Supplementary Fig. 5b), indicating that GABA is excitatory in granule cells migrating in the hilus. Neither AMPA nor NMDA evoked currents in GFP<sup>+</sup> cells in the hilus ( $n = 3$ ; Fig. 2j), whereas they both evoked inward currents in GFP<sup>+</sup> cells in the GCL ( $n = 3$ , Supplementary Fig. 5c). Thus, neonatally generated granule cells

**Figure 3** GABA<sub>A</sub>-R hyperactivity underlies aberrant granule cell migration. (a) Diagrams for the migration assay (top) and the trajectories of granule cells for 6 h (bottom). The trajectories of reversed migration are shown in red. (b) Cumulative frequency of the rate of movement of the granule cells.  $^{***}P < 0.01$  by Kolmogorov-Smirnov test ( $n = 22$ –28 cells per group). (c) Time-lapse (left) and immunostained (right) images of an aberrantly migrated granule cell in a hyperthermic to normothermic coculture. Arrows point to the soma, and arrowheads point to the leading process. (d) A representative ectopic granule cell (arrow) in a hyperthermic to normothermic coculture at 12 DIV. (e, f) Images of the explant culture (e) and immunostained images of migrating granule cells (f). (g–i) Immunocytochemical (ICC; g) and immunoblot (IB; h) images and quantification (i) of the surface expression of the  $\beta$  subunits of GABA<sub>A</sub>-Rs, which were assessed by using antibodies specific to the extracellular domain of the  $\beta$  subunits of the GABA<sub>A</sub>-Rs. p85 was used as a loading control. Error bars (i), s.e.m. MW, molecular weight.



initially receive GABA-mediated tonic currents during migration and thereafter receive glutamate-mediated synaptic inputs when they reach the GCL, similar to adult-born granule cells<sup>33</sup>.

### Aberrant migration of granule cells after febrile seizures

We next designed a slice coculture system in which we prepared GFP<sup>+</sup> hilar grafts and wild-type host slices from P12 rats in the normothermic and the hyperthermic groups (24 h after the induction of febrile seizures) (Fig. 3a). A time-lapse analysis revealed that 12 of the 26 GFP<sup>+</sup> cells from the hyperthermic grafts migrated in the opposite direction in host normothermic slices (hyperthermic to normothermic cocultures) (Fig. 3a–c and Supplementary Video 3). In contrast, 26 of 28 cells and 23 of 25 cells from the normothermic to normothermic and normothermic to hyperthermic cocultures, respectively, migrated correctly toward the GCL ( $P < 0.01$  between normothermic to normothermic and hyperthermic to normothermic;  $P = 0.65$  between normothermic to normothermic and normothermic to hyperthermic by Fisher's exact probability test) (Fig. 3a). We found ectopic granule cells in the hyperthermic to normothermic cocultures at 12 DIV (Fig. 3d). Administration of bicuculline prevented the aberrant migration of the granule cells and increased the rate of migration ( $n = 23$ ; Fig. 3a,b).

What functional changes are induced by febrile seizures in migrating granule cells? Febrile seizures did not affect the expression of glutamate decarboxylase (GAD)-67 or GABA in the dentate gyrus (Supplementary Fig. 6). We therefore isolated the hilar explants from P12 rats in either the normothermic or hyperthermic group (24 h after the induction of febrile seizures) and cultured them for 48 h (Fig. 3e). We found a large number of Prox1<sup>+</sup> granule cells with a polarized morphology typical of migrating neurons<sup>34</sup> around the explants (Fig. 3f). The immunocytochemical staining revealed that the surface expression of GABA<sub>A</sub>-R  $\beta$  subunits was upregulated in cells from the rats in the hyperthermic group (Fig. 3g). We confirmed this upregulation by immunoblotting plasma membrane proteins extracted from the hilar slices (Fig. 3h). The quantification of these results is shown in Figure 3i.

Puff application of muscimol to the growth cones of the leading processes of migrating cells caused a reversal in the direction of the migrating hyperthermic cells but not the migrating normothermic cells (Fig. 4a–c and Supplementary Videos 4 and 5), suggesting an increased sensitivity of hyperthermic granule cells to GABA.

We analyzed the cell positions relative to the explants after 72 h *in vitro* (Fig. 4d,e). The cell migration rate was suppressed by muscimol, an effect that was stronger in the hyperthermic granule cells than in the normothermic cells (Fig. 4f). This effect was blocked by an inhibitor of L-type Ca<sup>2+</sup> channels (1  $\mu\text{M}$  nimodipine). Thus, depolarization-induced Ca<sup>2+</sup> entry may mediate migration deficits through Ca<sup>2+</sup>-dependent cytoskeletal reorganization<sup>36,35–37</sup>.

The excitatory action of GABA on immature neurons is mediated by the accumulation of Cl<sup>−</sup> through the NKCC1 transporter (also known as SLC12A2)<sup>38,39</sup>. Indeed, a selective inhibitor of NKCC1 (10  $\mu\text{M}$  bumetanide) prevented muscimol-induced migration deficits (Fig. 4f). To further confirm the involvement of NKCC1, we knocked down NKCC1 in hyperthermic granule cells using NKCC1-targeted shRNA. At 48 h after knockdown, the amount of NKCC1 protein was reduced in the cells transfected with NKCC1-targeting shRNA but not in control cells transfected with nontargeting shRNA (Supplementary Fig. 7). The NKCC1 knockdown rescued GABA<sub>A</sub>-R-mediated migration deficits (Fig. 4g,h).

### Bumetanide rescues granule cell ectopia and epilepsy

In the *in vivo* febrile seizure model, bumetanide also prevented granule cell ectopia (Fig. 5a,b). Neither febrile seizures nor bumetanide attenuated the dendritic maturation of neonatally (P5)-generated granule cells in the GCLs at P60 (Supplementary Fig. 8), although NKCC1 has been reported to contribute to dendritic maturation in adult-born granule cells<sup>40</sup>.

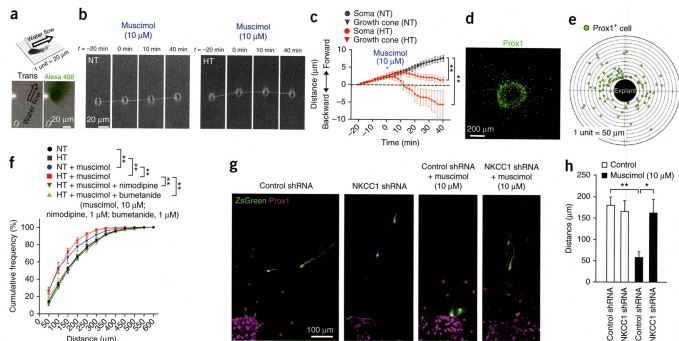


**Figure 4** NKCC1 inhibition rescues granule cell migration deficits and ectopia. (a–c) Time-lapse analysis of migrating granule cells before and after muscimol application at 48 h in the explant culture. Muscimol was focally applied to the leading growth cones (a).  $^{**}P < 0.01$  by Student's *t* test ( $n = 4$ –5 cells per group). (d,e) Immunostained (d) and plotted (e) images of granule cells at 72 h in the explant culture. (f) Cumulative frequency of the migration distance. Reagents were applied between 24 h and 72 h in explant culture.  $^{**}P < 0.01$  by Kolmogorov–Smirnov test ( $n = 5$ –6 explants per group). (g,h) Immunostained images (g) and mean migration distance (h) of shRNA-transfected hyperthermic granule cells cultured with or without muscimol. Error bars, s.e.m.  $^{*}P < 0.05$ ,  $^{**}P < 0.01$  by Tukey's test after one-factor ANOVA ( $n = 13$ –19 cells per group).

To examine whether febrile seizures in early life increase the susceptibility to seizures in adulthood, we treated P60 rats with a subconvulsive dose of pilocarpine (300 mg per kg body weight). Pilocarpine induced limbic seizures that persisted for >80 min in rats in the

hyperthermic group but not in the rats in the normothermic and hyperthermic control groups (Fig. 5c). This increased susceptibility to adult seizures was blocked by the application of bumetanide after febrile seizures at P11. The maximal average behavioral score

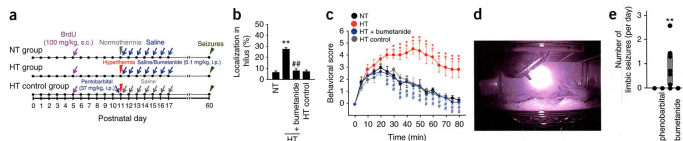


**Figure 5** Bumetanide rescues granule cell ectopia, seizure susceptibility and development of epilepsy. (a) Experimental paradigms. (b) Localization (%) of neonatally (P5)-generated granule cells in the hilus at P60. Error bars, s.e.m.  $^{**}P < 0.01$  compared to normothermic;  $^{*}P < 0.01$  by Tukey's test after one-factor ANOVA compared to hyperthermic ( $n = 16$  sections from four rats per group). (c) Seizure scores every 5 min for 80 min after pilocarpine injection from rats in each experimental group (P60).  $^{**}P < 0.01$  compared to normothermic,  $^{*}P < 0.01$  by Bonferroni test compared to hyperthermic in the same time bins ( $n = 6$  rats per group). (d) Still shot from a movie of a seizing adult rat that experienced febrile seizures (from the hyperthermic group). Rats with or without febrile seizures were observed after postnatal weeks 12 to 13. (e) Spontaneous limbic seizures (Online Methods) were observed only in rats from the hyperthermic group ( $n = 16$ ) but not in the rats from the normothermic ( $n = 20$ ), normothermic plus phenobarbital ( $n = 14$ ) or hyperthermic plus bumetanide ( $n = 15$ ) groups.  $^{**}P < 0.01$  compared to normothermic by Steel–Dwass test after Kruskal–Wallis test. (f) Rats with seizures ( $n = 8$ ) had larger numbers of ectopic granule cells compared to rats without seizures ( $n = 8$ ) in the hyperthermic group. Error bars, s.e.m.  $^{*}P < 0.05$  by Student's *t* test. (g) Box plots showing the relationship between the number of ectopic granule cells and the seizure frequency per day. A total of 16 rats in the hyperthermic group were categorized into two groups according to the number of ectopic granule cells, high: top 50%,  $n = 8$ ; low: bottom 50%,  $n = 8$ .  $^{*}P < 0.05$  by Mann–Whitney *U* test. (h) Correlation between the ectopic granule cell number and the seizure frequency ( $n = 16$  rats) in the hyperthermic group.  $R = 0.31$ ,  $P < 0.05$  by ANOVA.

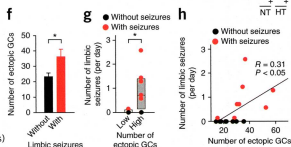
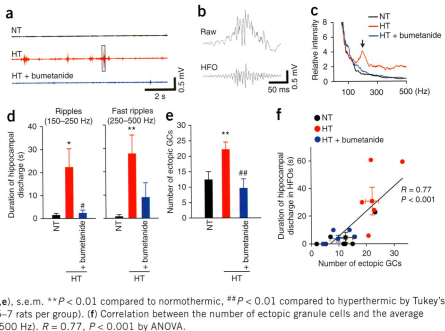




**Figure 6** Bumetanide blocks the emergence of ectopic granule cells and epileptic ripples in adulthood. **(a)** Representative LFP traces recorded from the CA1 pyramidal cell layer of rats from the three experimental groups. **(b)** Magnification of the boxed region in **a** confirming that the hyperactivity observed in a raw LFP trace (top) consisted of an HFO (bottom; 150–500 Hz). **(c)** Representative spectrograms from the three experimental groups. The oscillation powers were normalized to the 200-Hz intensity in the normothermic group. The rats in the hyperthermic group had a spectrum peak at approximately 200 Hz (arrow). Experiments in **a–c** were reproduced at least three times, and similar results were obtained in each. **(d)** The mean duration of hippocampal discharges in ripples (left; 150–250 Hz) and fast ripples (right; 250–500 Hz). \* $P < 0.05$ , \*\* $P < 0.01$  compared to normothermic, # $P < 0.05$  compared to hyperthermic by Tukey's test after one-factor ANOVA ( $n = 5$ –7 rats per group). **(e)** The number of ectopic granule cells in each group. Error bars (**d,e**), s.e.m. \*\* $P < 0.01$  compared to normothermic, ## $P < 0.01$  compared to hyperthermic by Tukey's test after one-factor ANOVA ( $n = 12$  sections from 5–7 rats per group). **(f)** Correlation between the number of ectopic granule cells and the average duration of hippocampal discharges in HFOs (150–500 Hz).  $R = 0.77$ ,  $P < 0.001$  by ANOVA.



was  $5.0 \pm 0.4$  (mean  $\pm$  s.e.m.) in the hyperthermic group and  $3.2 \pm 0.2$  in the hyperthermic plus bumetanide group ( $n = 6$  rats in each). Specifically, we observed head bobbing (corresponding to a score of 4, two rats), rearing and forelimb clonus (corresponding to a score of 5, two rats) and rearing, clonus and falling (corresponding to a score of 6, two rats) in the hyperthermic group, whereas only one of the six rats in each of the normothermic, hyperthermic plus bumetanide and hyperthermic control groups reached a score of 4.

We killed the rats 80 min after the injection of pilocarpine and evaluated the activity of their granule cells using expression of c-Fos, an immediately early gene product that is rapidly induced by neuronal activity and can be used as a marker of the activity history of the neurons<sup>41</sup> (Supplementary Fig. 9a,b). The rats in the hyperthermic group had more c-Fos<sup>+</sup> granule cells in the hilus and in the GCL than the other groups (Supplementary Fig. 9c,d). Thus, ectopic granule cells in the hyperthermic group were activated during limbic seizures, which may recruit the recurrent activation of normal granule cells, resulting in behavioral seizures.

We carried out long-term video monitoring (9 p.m. to 5 a.m. for 7 successive days) of 12- to 13-week-old rats that had experienced febrile seizures and compared their behavioral profiles to the number of ectopic granule cells. Eight out of 16 rats in the hyperthermic group showed spontaneous limbic seizures consisting of rearing and bilateral clonuses, which were sometimes followed by falling (Fig. 5d). These seizures are known to accompany electrophysiological seizures in the hippocampus in the same febrile seizure models<sup>23</sup>. None of the rats in the normothermic, normothermic plus phenobarbital or hyperthermic plus bumetanide groups developed spontaneous seizures (Fig. 5e). The rats in the hyperthermic group with spontaneous seizures had more ectopic granule cells than the rats in the hyperthermic group without spontaneous seizures (Fig. 5f,g). The number of ectopic granule cells correlated positively with the frequency of spontaneous seizures in the hyperthermic group (Fig. 5h). This correlation was also previously reported in the adult pilocarpine model of TLE<sup>42</sup>.

We conducted long-term LFP recordings from the CA1 region of adult rats (9–11 weeks old) that had experienced febrile seizures.

We focused on the high-frequency oscillations (HFOs) with spectral frequencies of 150–500 Hz because the dentate gyrus and CA1 in a TLE model show abnormal discharges in this oscillation band<sup>43</sup>. Rats in the hyperthermic group showed hyperactivity in their HFOs, which were sustained over 20 s (Fig. 6a,b), whereas rats from the normothermic and hyperthermic plus bumetanide groups did not (Fig. 6c). We separately analyzed these aberrant HFOs in ripples (150–250 Hz) and fast ripples (250–500 Hz); the latter is suggested to serve as a pathological marker<sup>44</sup>, although recent findings have suggested that the former is also increased in seizure-generating brain regions<sup>45</sup>, making it difficult to distinguish normal and pathological HFOs by the oscillation frequency alone<sup>46</sup>. Indeed, rats in the hyperthermic group showed hyperactivity in both ripples and fast ripples (Fig. 6d). Bumetanide prevented febrile-seizure-induced increases in HFOs (but not significantly in fast ripples;  $P = 0.078$ , Tukey's test after one-factor analysis of variance (ANOVA)) (Fig. 6d), as well as in the number of ectopic granule cells (Fig. 6e) in the hyperthermic group. In the pooled data from the rats in all the groups together, the duration of hippocampal hyperactivity correlated positively with granule cell ectopia (Fig. 6f).

## DISCUSSION

It has been unclear whether and how complex febrile seizures induce pathophysiological alternations in the developing brain and how they are related to epilepsy later in life<sup>40</sup>, mainly because of the limitations of human studies. Here we have addressed these issues by using an animal model of febrile seizures<sup>19,21–23</sup> and developing an *in vitro* system that can replicate febrile-seizure-induced anatomical changes. We discovered that excitatory GABA<sub>A</sub>-R signaling is enhanced after febrile seizures and disturbs the radial migration of neonatally generated granule cells, resulting in persistent granule cell ectopia. Furthermore, we show a statistical correlation between ectopic granule cells and epilepsy in adult rats that experienced febrile seizures. These results suggest that blockade of excitatory GABA<sub>A</sub>-R signaling in the developing brain is a possible therapeutic target for febrile seizures and that at this early stage, medications that enhance

GABA<sub>A</sub>-R function, such as barbiturates and benzodiazepines that may be administered to children with febrile seizures, could induce aberrant maturation of dentate neuronal circuits. However, our results must be extrapolated to clinical treatment with caution, as it is unclear whether phenobarbital, a GABA<sub>A</sub>-R agonist, or other drugs that enhance GABA<sub>A</sub>-R function increase the risk of developing epilepsy in humans or rodent models. Although we did not simultaneously monitor behavioral and electroencephalographic seizures, multisite recordings within and outside the dentate gyrus in combination with behavioral monitoring over longer time periods would allow us to more clearly distinguish epileptic and nonepileptic events and specify the role of ectopic granule cells in initiating or propagating the ictal activity that leads to seizures.

We found that febrile-seizure-induced excitatory GABA<sub>A</sub>-R signaling resulted in a reversal in the direction of migration through an influx of Ca<sup>2+</sup>. In cerebellar neurons, Slit-2-induced intracellular Ca<sup>2+</sup> waves have previously been shown to change the orientation of the leading process from forward to backward by redistribution of RhoA, a member of the Rho family of GTPases, and thereby reverse the direction of migration<sup>36</sup>.

Our results are also in agreement with previous studies regarding cortical neuronal migration<sup>29,47</sup> and suggest that ambient GABA inhibits cell migration through activation of GABA<sub>A</sub>-R. Under normal conditions, this role of GABA may underlie the proper layering of granule cells by incorporating newborn granule cells into the innermost region of the GCL<sup>48</sup>. Possible sources of GABA include interneurons and hippocampal mossy fibers, which release GABA in young animals<sup>49</sup> and after seizures<sup>50</sup>. It will also be necessary to determine whether the migrating granule cells release GABA and communicate with each other to regulate migration speed, as has been reported in tightly migrating subventricular-zone neuroblasts<sup>28</sup>.

We here provide evidence that granule cell ectopia, susceptibility to limbic seizures and the development of epilepsy in adulthood are all prevented by a widely used loop diuretic, bumetanide<sup>51</sup>, even after febrile seizures. Bumetanide is known to decrease seizure activity in neonatal rats<sup>52</sup> and in a human neonate<sup>53</sup>. Thus, further drug designs are required to increase the permeability of bumetanide through the blood-brain barrier, although the clinical evidence for use of bumetanide for febrile seizure treatment is not yet available.

Our study does not exclude the possibility that mechanisms that are unrelated to granule cell ectopia underlie the long-lasting modulation of hippocampal network functions after febrile seizures. For example, potentiated inhibitory inputs and larger hyperpolarization-activated depolarizing currents (*I<sub>h</sub>*) could contribute synergistically to the febrile-seizure-induced hyperexcitability of CA1 pyramidal neurons<sup>21,22,54</sup>. Other neuroanatomical changes, such as mossy fiber sprouting and loss of inhibitory interneurons<sup>20</sup>, could also contribute to hippocampal hyperactivity on their own or in combination with ectopic granule cells. Indeed, we did not find spontaneous seizures in phenobarbital-injected rats, even though these rats had a higher number of ectopic granule cells than noninjected rats, indicating that ectopic granule cells alone are not sufficient to cause epilepsy in our experimental paradigm. Further studies are necessary to examine whether ectopic granule cells and other alterations act synergistically to generate persistent hyperexcitable foci in the hippocampus that cause epilepsy.

## METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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## AUTHOR CONTRIBUTIONS

R.K. designed the project, conducted and analyzed the experiments, and wrote the manuscript. K.T. did *in vivo* electrophysiology, long-term video monitoring and helped with preparation of retroviruses. T.S. did *in vitro* electrophysiology and time-lapse imaging in explant cultures. J.I. did injection of retroviruses. D.M. helped with *in vivo* electrophysiology. R.M. helped with perfusion of mice. N.M. and Y.I. are senior authors and were responsible for project planning. All authors analyzed and discussed the results and commented on the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance

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Toll-like receptor 4 (TLR4) has a key role in innate immunity by activating an inflammatory signaling pathway. Free fatty acids (FFAs) stimulate adipose tissue inflammation through the TLR4 pathway, resulting in insulin resistance<sup>1–7</sup>. However, current evidence suggests that FFAs do not directly bind to TLR4<sup>8,9</sup>, but an endogenous ligand for TLR4 remains to be identified. Here we show that fetuin-A (FetA) could be this endogenous ligand and that it has a crucial role in regulating insulin sensitivity via Tlr4 signaling in mice. *FetA* (officially known as *Ahsg*) knockdown in mice with insulin resistance caused by a high-fat diet (HFD) resulted in downregulation of Tlr4-mediated inflammatory signaling in adipose tissue, whereas selective administration of FetA induced inflammatory signaling and insulin resistance. FFA-induced proinflammatory cytokine expression in adipocytes occurred only in the presence of both FetA and Tlr4; removing either of them prevented FFA-induced insulin resistance. We further found that FetA, through its terminal galactoside moiety, directly binds the residues of Leu100–Gly123 and Thr493–Thr516 in Tlr4. FFAs did not produce insulin resistance in adipocytes with mutated Tlr4 or galactoside-cleaved FetA. Taken together, our results suggest that FetA fulfills the requirement of an endogenous ligand for TLR4 through which lipids induce insulin resistance. This may position FetA as a new therapeutic target for managing insulin resistance and type 2 diabetes.

Insulin resistance is one of the major outcomes of chronic inflammation fueled by FFAs<sup>10–13</sup>. There has been growing evidence over the last few years that FFA-induced production of proinflammatory cytokines from inflamed adipose tissue is mediated through TLR4, a pattern recognition receptor. FFA-mediated activation of the TLR4 and nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathways has been increasingly recognized as a cause of insulin resistance<sup>4–8</sup>; however, in the absence of a direct association between FFAs and the TLR4–MD2 complex<sup>8,9</sup>, how FFAs activate TLR4 signaling remains unresolved. This has created a crucial deficiency in the understanding of the regulation of the

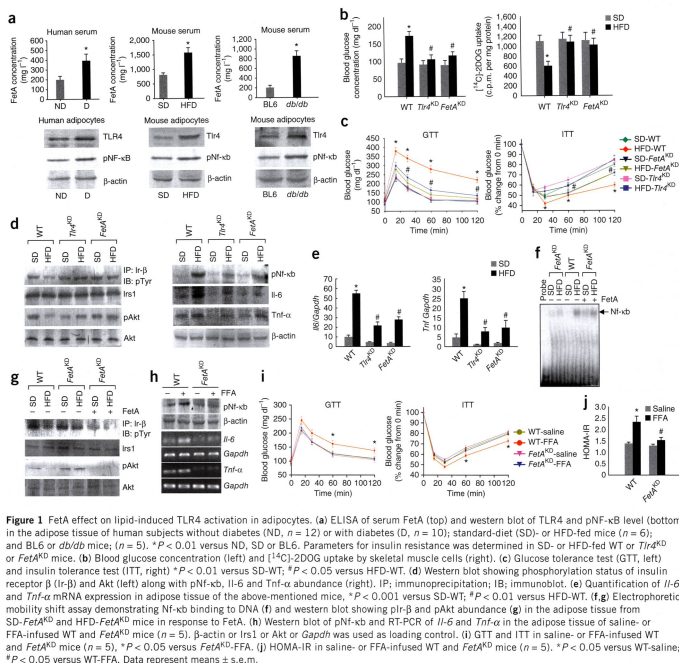
FFA–TLR4 signaling pathway. Identification of an endogenous ligand that allows FFA–TLR4 crosstalk is therefore crucial to our understanding of the mechanism by which FFAs induce insulin resistance.

FetA, a liver secretory glycoprotein, stimulates the production of inflammatory cytokines from adipocytes and macrophages and therefore acts as a biomarker of chronic inflammatory diseases<sup>14–16</sup>. We have demonstrated that FFAs cause FetA overexpression through NF- $\kappa$ B<sup>17</sup> and that elevation of FetA's circulatory level enhanced proinflammatory cytokine production from adipocytes<sup>17,18</sup>. Notably, *FetA*- or *Tlr4*-knockout mice are protected from HFD-induced insulin resistance<sup>4,19–21</sup> and it has also been reported that FetA acts as a major carrier protein of FFAs in the circulation<sup>22</sup>. On the basis of these reports, we hypothesized that FetA may present FFAs to TLR4 by its actions as an endogenous ligand for this receptor.

To examine our hypothesis, we determined FetA concentration in serum, as well as *Tlr4*, *IL-6* and *TNF- $\alpha$*  expression and NF- $\kappa$ B activation in adipocytes, isolated from obese diabetic human subjects, HFD-induced insulin resistant mice and dyslipidemic *db/db* mice. We found that all of these parameters were significantly elevated in these conditions in comparison with nonobese nondiabetic human subjects, standard diet (SD)-fed BALB/c mice and wild-type BL/6 mice, respectively (Fig. 1a and Supplementary Fig. 1a). This suggests an association between lipid, FetA concentration and TLR4 expression and activation in states of insulin resistance.

As *FetA*<sup>−/−</sup> and *Tlr4*<sup>−/−</sup> mice are protected from lipid-induced insulin resistance<sup>4,19–21</sup>, we thought that using them to examine FetA–TLR4 interdependence in producing insulin resistance might not be useful. We therefore prepared diet-induced insulin-resistant mice and then used *vivo*-morpholino (VMO)-based gene knockdown of *FetA* or *Tlr4* (*FetA*<sup>KD</sup> or *Tlr4*<sup>KD</sup> mice); we verified knockdown efficiency by real-time quantitative PCR (qPCR), but knockdown did not significantly alter body and adipose tissue weight (Supplementary Fig. 1b,c), as this would be an ideal model to understand the FetA–TLR4 association in implementing insulin resistance. *FetA*<sup>KD</sup> or *Tlr4*<sup>KD</sup> mice were protected from insulin resistance due to a HFD, as was evident from differences in blood glucose levels, [<sup>14</sup>C]-2-deoxy-D-glucose ([<sup>14</sup>C]-2DOG) uptake, responses to

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a glucose tolerance test (GTT) and an insulin tolerance test (ITT), homeostasis model assessment–insulin resistance (HOMA-IR) scores and phosphorylation of the insulin–signaling molecules insulin receptor- $\beta$  (Ir- $\beta$ ) and Akt (Fig. 1b–d and Supplementary Fig. 1d), suggesting that both FetaA and Tlr4 are necessary for lipid-mediated insulin resistance in our mouse model. This was further indicated by the reduced NF- $\kappa$ B activity along with the marked reduction of interleukin-6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) mRNA expression in adipocytes from *Feta*<sup>KD</sup> and *Tlr4<sup>KD</sup> mice as compared to wild-type mice under the same dietary regimen. (Fig. 1e).*

The involvement of TLR4 in lipid-induced insulin resistance has been shown previously<sup>4–7</sup>, therefore it was expected, but we were intrigued by the similar trend of the results in *Feta*<sup>KD</sup> mice. Thus, to validate our findings further, we introduced FetaA, free of endotoxin

(a TLR4 ligand expressed by bacteria and hence a common contaminant in recombinant proteins isolated from bacterial expression systems) (Supplementary Fig. 1e–h) to *Feta*<sup>KD</sup> mice and found this manipulation resulted in lipid-induced insulin resistance (Fig. 1f,g and Supplementary Fig. 1d).

As the liver is a major source of FetaA, we partially hepatectomized rats to lower the serum levels of FetaA as an alternative means to genetically knocking down the expression of the protein. We observed a significant decrease in serum FetaA levels along with a reduction in pNF- $\kappa$ B, IL-6 and TNF- $\alpha$  in partially hepatectomized HFD rats as compared to control HFD rats (Supplementary Fig. 1i).

Adipose tissue from lipid-infused *Feta*<sup>KD</sup> mice did not show Tlr4 activation, whereas it was apparent in wild-type (WT) mice (Fig. 1h). Glucose and insulin tolerances were decreased, and



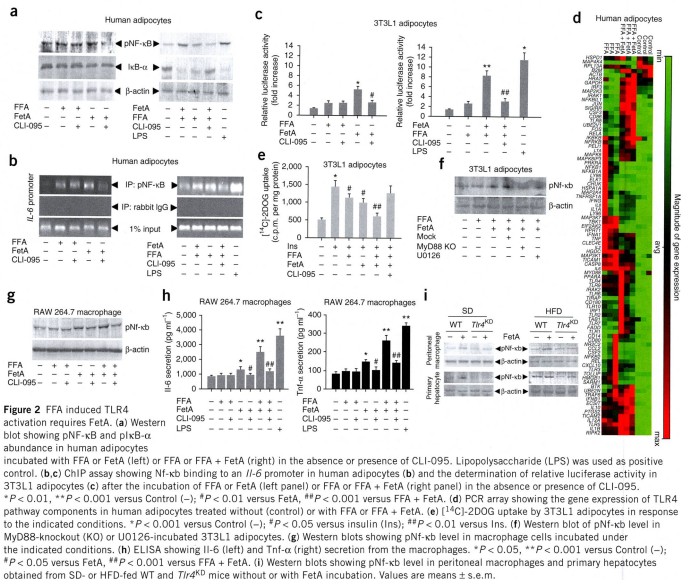


**Figure 2** FFA induced TLR4 activation requires FetA. **(a)** Western blot showing pNF- $\kappa$ B and pI $\kappa$ B- $\alpha$  abundance in human adipocytes incubated with FFA or FetA (left) or FFA or FFA + FetA (right) in the absence or presence of CLI-095. Lipopolysaccharide (LPS) was used as positive control. **(b,c)** ChIP assay showing NF- $\kappa$ B binding to an *IL-6* promoter in human adipocytes **(b)** and the determination of relative luciferase activity in 3T3L1 adipocytes **(c)** after the incubation of FFA or FetA (left panel) or FFA or FFA + FetA (right panel) in the absence or presence of CLI-095. \* $P < 0.01$ , \*\* $P < 0.001$  versus Control (-); \* $P < 0.01$  versus FetA, \*\* $P < 0.001$  versus FFA + FetA. **(d)** PCR array showing the gene expression of TLR4 pathway components in human adipocytes treated without (control) or with FFA or FFA + FetA. **(e)** [ $^{125}$ I]-2DGO uptake by 3T3L1 adipocytes in response to the indicated conditions. \* $P < 0.001$  versus Control (-); \*\* $P < 0.05$  versus insulin (Ins); \*\* $P < 0.001$  versus Ins. **(f)** Western blot of pNF- $\kappa$ B level in MyD88-knockout (KO) or U0126-incubated 3T3L1 adipocytes. **(g)** Western blots showing pNF- $\kappa$ B level in macrophage cells incubated under the indicated conditions. **(h)** ELISA showing IL-6 (left) and TNF- $\alpha$  (right) secretion from the macrophages. \* $P < 0.05$ , \*\* $P < 0.001$  versus Control (-); \* $P < 0.05$  versus FetA, \*\* $P < 0.001$  versus FFA + FetA. **(i)** Western blots showing pNF- $\kappa$ B level in peritoneal macrophages and primary hepatocytes obtained from SD- or HFD-fed WT and *Tlr4*<sup>KO</sup> mice without or with FetA incubation. Values are means  $\pm$  s.e.m.

insulin resistance was increased after palmitate infusion in WT mice, whereas in *Tlr4*<sup>KO</sup> mice these parameters were unchanged (Fig. 1j and Supplementary Fig. 1j).

These results implicate three important possibilities regarding the involvement of FetA and Tlr4 in lipid-induced insulin resistance: (i) the decrease in NF- $\kappa$ B phosphorylation in the absence of FetA may be due to impaired Tlr4 signaling, (ii) insulin resistance due to lipotoxicity requires the presence of both Tlr4 and FetA, and (iii) a physical association between FFAs, FetA and Tlr4 is likely to be linked to insulin resistance.

To address the first possibility, we investigated whether FFA-induced Tlr4 signaling requires FetA. Palmitate (a FFA) had maximum binding to FetA and produced the highest inflammatory response in 3T3L1 mouse adipocytes as compared to other FFAs (Supplementary Fig. 2a,b). We therefore incubated human adipocytes or 3T3L1 cells with palmitate or FetA or both of them in the absence or presence of CLI-095, a TLR4 signaling inhibitor, followed by the determination of NF- $\kappa$ B activity through western blotting, chromatin immunoprecipitation (ChIP) and reporter assays. FFA-induced activation of NF- $\kappa$ B and elevated expression of the cytokines *IL-6* and *TNF- $\alpha$*  occurred

only in the presence of FetA, whereas CLI-095 incubation or silencing of Tlr4 expression by siRNA prevented this activation and cytokine expression (Fig. 2a–c and Supplementary Fig. 2c,d).

These results were notable given previous reports suggesting that FFAs activate the Tlr4 signaling pathway in 3T3L1 adipocytes<sup>24,6,23,24</sup>. One possibility for this disparity could be the high FetA concentration in FBS<sup>25</sup>. As commercially available FBS contains ~20 mg ml<sup>-1</sup> FetA, conventionally used culture media (with 10% FBS) have a substantial amount of FetA, which might allow exogenously added FFAs to induce Tlr4 activation. We therefore used serum-free medium in our experiments. FFAs alone had a marginal stimulatory effect that seemed to be independent of Tlr4 because addition of CLI-095 to the medium did not alter the response to exogenous FFAs (Fig. 2a–c). That FFA can induce inflammatory signaling pathways independent of Tlr4 influence has also been shown by others<sup>26,27</sup>. In contrast, stimulation of NF- $\kappa$ B activation by FetA was inhibited by CLI-095, indicating that FetA's effect is mediated through Tlr4 (Fig. 2a–c).

We made similar observations when we incubated macrophages and adipocytes obtained from *Tlr4*<sup>-/-</sup> mice (Tlr4 knockout was verified by RT-PCR, Supplementary Fig. 2e) with FetA and saw that FetA did

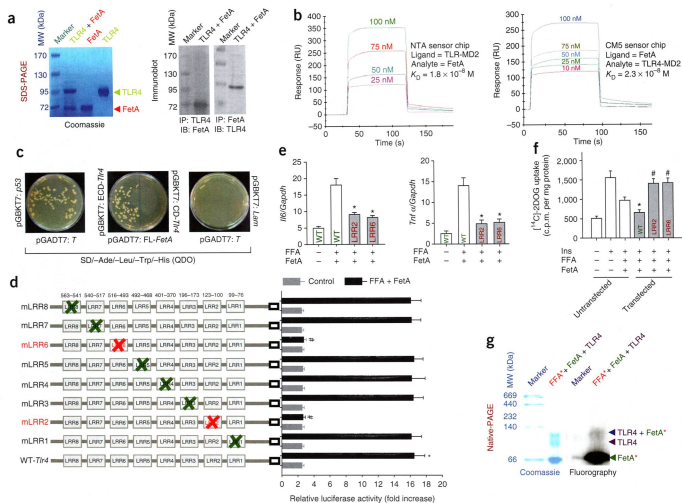


**Figure 3** FcTA-TLR4 interaction induces proinflammatory cytokine expression and insulin resistance in adipocytes. **(a)** SDS-PAGE (left) and Western blot (right) of coimmunoprecipitation study demonstrating FcTA binding to TLR4. **(b)** SPR study showing representative sensorgrams obtained from flowing of the indicated concentrations of FcTA (left) or TLR4-MD2 (right) over the TLR4-MD2 immobilized NTA sensor chip or FcTA immobilized CM5 sensor chip, respectively. Sensorgrams are represented as response units (RU) at specified time (s). **(c)** Y2H showing FcTA binding with the extracellular domain (ECD) of TLR4 as indicated by the growth colonies in growth-limiting plates. pGADT7::T and pGBKT7::p53 plasmids and pGADT7::T and pGBKT7::Lam plasmids were used as positive and negative controls, respectively. **(d)** Schematic structure showing the eight predicted LRR regions in the ECD of TLR4. These were individually mutated by site-directed mutagenesis (left), and transfected 3T3L1 adipocytes were examined for NF- $\kappa$ B luciferase activity (right). \* $P < 0.001$  versus control (-); \*\* $P < 0.001$  versus WT Tlr4. **(e)** Quantification of *Il-6* and *Tnf- $\alpha$*  mRNA expression. \* $P < 0.01$  versus WT Tlr4. **(f)** [<sup>14</sup>C]-2DOG uptake in WT, LRR2-mutated and LRR6-mutated Tlr4-transfected 3T3L1 adipocytes. \* $P < 0.05$  versus WT Tlr4 untransfected; \*\* $P < 0.01$  versus WT Tlr4 transfected. **(g)** Representative gels of native-PAGE and its fluorography showing formation of a ternary complex between FFA, FcTA and TLR4 (asterisks represent [<sup>3</sup>H]-palmitate and its association with FcTA). Data represent means  $\pm$  s.e.m.

not activate the Tlr4 pathway, thus indicating the requirement of Tlr4 for FcTA's effect on NF- $\kappa$ B activation (Supplementary Fig. 2f). We also evaluated FFA plus FcTA (FFA+FcTA) induction of TLR4 signaling by PCR array and found that gene expression of various proinflammatory cytokines in human adipocytes belonging to the TLR4 pathway were greatly upregulated, whereas FFA alone produced a marginal effect (Fig. 2d). This was also reflected in the insulin sensitivity assay, wherein FFA+FcTA showed a significantly greater inhibitory effect on insulin-stimulated [<sup>14</sup>C]-2DOG uptake by 3T3L1 adipocytes as compared to FFA or FcTA alone (Fig. 2e).

Because myeloid differentiation primary response gene 88 (MyD88) and mitogen-activated protein kinase kinase (MEK) are downstream of the TLR4 signaling pathway<sup>28,29</sup>, failure of NF- $\kappa$ B activation in MyD88-knockout 3T3L1 adipocyte cells incubated with FFA+FcTA

or in cells preincubated with the MEK inhibitor U0126 (Fig. 2f) suggest that FFA+FcTA acts through the TLR4 pathway. We found that FFA+FcTA experiments with macrophages also had similar results; FFA augmentation of Tlr4 activation was dependent on FcTA (Fig. 2g,h), as observed with adipocytes, indicating a link between insulin resistance and innate immunity with FFAs as reported earlier<sup>4</sup>. We examined this further in primary cell cultures of macrophage and hepatocytes obtained from standard diet-fed *Tlr4*<sup>KD</sup> mice, in which FcTA alone failed to induce NF- $\kappa$ B activation but did so in standard diet-fed WT mice (Fig. 2i). We obtained similar results with HFD-fed insulin-resistant mice (Fig. 2j), suggesting that Tlr4 is necessary to produce the FFA+FcTA effect on NF- $\kappa$ B activation.

From these results it would seem evident that FcTA is required for FFA-TLR4 signaling. However, we along with others have observed

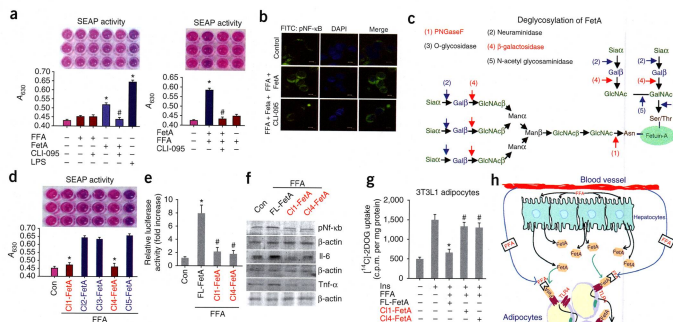


**Figure 4** Cleavage of terminal  $\beta$ -galactosides of FetA fails to stimulate TLR4 pathway. (a) TLR4 activation in terms of SEAP activity in TLR4-MD2-overexpressing HEK-Blue hTLR4 cells in response to FFA or FetA (left) or FFA or FFA + FetA (right) in the presence or absence of CL1-095. LPS served as positive control. \* $P < 0.001$  versus control (-); # $P < 0.01$  versus FetA or FFA + FetA. (b) Immunostaining showing localization of pNf- $\kappa$ B (green) and DAPI (blue) in HEK-Blue hTLR4 cells treated under the indicated conditions. Scale bars, 5  $\mu$ m. (c) Schematic representation of cleavage sites in N-linked and O-linked glycan moieties of FetA are indicated by red and blue arrows. Sia, sialic acid; Gal, galactose; Man, mannose; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; Asn, Asparagine, Ser/Thr, Serine/Threonine. (d) Inhibition of SEAP activity in response to cleaved FetAs (C1 and C14) by PNGaseF and  $\beta$ -galactosidase enzymes. \* $P < 0.001$  versus C12, C13 and C15. (e) Inhibition of NF- $\kappa$ B reporter activity in 3T3L1 adipocytes treated with FFA plus C1 or C14 FetA. \* $P < 0.001$  versus Control (-); # $P < 0.01$  versus full-length FetA (FL-FetA). (f) Western blot showing pNf- $\kappa$ B, I- $\kappa$ B and Tnf- $\alpha$  abundance in 3T3L1 adipocytes (f) and [ $^{14}$ C]-2DOG uptake by 3T3L1 adipocytes (g) incubated with FL or cleaved FetAs (C1 and C14). \* $P < 0.01$  versus FL-FetA. Values are means  $\pm$  s.e.m. (h) Proposed model highlighting the requirement of FetA in lipid-induced TLR4 activation that leads to insulin resistance. Error bars represent mean  $\pm$  s.e.m.

that FFAs have no direct association with TLR4 (refs. 8,9), whereas they bind FetA appreciably<sup>22</sup> (Supplementary Fig. 3a). We found further evidence of these facts in a lipid-protein overlay assay where FFAs bound FetA but not TLR4 (Supplementary Fig. 3b). Hence, it is possible that FetA acts as an intermediary between FFA and TLR4. Indeed, coimmunoprecipitation revealed the existence of a complex between FetA and TLR4 (Fig. 3a and Supplementary Fig. 3c).

To study the nature of this complex and to probe the possibility of a physical interaction between FetA and TLR4 as proposed above, we performed surface plasmon resonance (SPR) in which varied concentrations of FetA were flowed over a histidine-tagged human TLR4-myeloid differentiation factor 2 (MD2) complex which was immobilized on a nitrilotriacetic acid (NTA) sensor chip. The resulting sensorgram showed concentration-dependent binding of FetA to TLR4, and the affinity of this binding was appreciably high, as indicated by its  $K_D$  value ( $1.8 \times 10^{-8}$  M). Reversing the situation, wherein FetA remained immobilized on the carboxymethylated dextran (CM5) chip and TLR4 was used as analyte, produced a similar binding affinity ( $K_D = 2.3 \times 10^{-8}$  M) (Fig. 3b). The inability of high mobility group A1 (HMGA1) to interact with TLR4 indicates the specificity of this interaction (Supplementary Fig. 3d).

To examine whether such a complex between FetA and TLR4 is operative in an intracellular system, we performed a yeast two-hybrid (Y2H) assay using pGBKT7 fused with DNA encoding the extracellular domain or cytosolic domain of TLR4. These plasmids were separately transfected into the Y2H Gold strain of yeast,

followed by mating with the Y187 strain containing full-length FetA inserted into pGADT7. FetA interacted with the extracellular domain of TLR4, as interaction-dependent growth of colonies was observed in SD/-Ade/-His/-Leu/-Trp (quadruple dropout, QDO) plates, whereas colony growth did not occur after mating with yeast expressing the plasmid encoding the cytoplasmic domain of TLR4 (Fig. 3c).

We then searched the probable sites of the TLR4 extracellular domain to which FetA binds. We presumed it could be the region of leucine-rich repeats (LRR), as those have been previously found to be involved in protein-protein interactions<sup>30</sup>. To analyze FetA recognition of specific LRR sites, we mutated different LRRs in TLR4. Deletion mutation of LRR2 (Leu100-Gly123) or LRR6 (Thr493-Thr516) in the extracellular domain of TLR4 impeded its interaction with FFA+FetA, as indicated by diminished NF- $\kappa$ B luciferase activity (Fig. 3d) and cytokine expression (Fig. 3e) in differentiated adipocytes and by  $\alpha$ -galactosidase activity in Y2H studies (Supplementary Fig. 3e). Deletion mutations at these sites not only restored but also augmented insulin sensitivity (Fig. 3f), possibly because of the dominant-negative nature of the mutations (Fig. 3d). Mutational studies suggest that these two LRRs of TLR4 are the crucial sites where FetA binds and permits FFAs to exert their deleterious effect on insulin activity. Hence, it is possible that FFAs, FetA and TLR4 all form one complex. To examine this, we incubated [ $^3$ H]-palmitate with FetA followed by human TLR4 incubation. Both Coomassie staining and fluorography of the native-PAGE gel revealed a ternary complex between the three of them (Fig. 3g).

To determine the biological relevance of FetA-TLR4 interactions in FFA-induced insulin resistance, we used genetically engineered HEK-Blue hTLR4 cells, which contain a TLR4–NF- $\kappa$ B–secreted embryonic alkaline phosphatase (SEAP) reporter system to detect TLR4–NF- $\kappa$ B activation. Incubation of FFAs in the presence of FetA enhanced SEAP activity, which was inhibited by CLI-095 (Fig. 4a), and this was dose and time dependent (Supplementary Fig. 4a–c), indicating that FFA–FetA operates through the TLR4 pathway. Immunofluorescence study further supported the notion that both FetA and TLR4 are required to transduce FFA signals, because FFA+FetA-stimulated nuclear translocation of NF- $\kappa$ B was abrogated by CLI-095 (Fig. 4b).

We identified the sequence of TLR4 where FetA binds; however, which part of FetA recognizes the TLR4 sequence remained unanswered. As terminal carbohydrate moieties of glycoproteins, such as sialic acid, galactose and fucose, underpin cell adhesion, receptor recognition and host–pathogen interactions<sup>31</sup>, we presumed that the glycan moieties of FetA bind TLR4, especially as Gram-negative bacteria trigger TLR4 signaling through the glycan residues of lipopolysaccharide<sup>32–34</sup>. To investigate this possibility, we used various enzymes to cleave specific glycosidic and amide bonds in FetA, as indicated (Fig. 4c). Truncated FetA and FetA's glycan part, obtained by enzymatic cleavage, were separately added to HEK-Blue hTLR4 cells.  $\beta$ -galactosidase- or PNGaseF-cleaved FetA was unable to stimulate SEAP activity (Fig. 4d and Supplementary Fig. 4d). Given that these results coincided with lower *Nf- $\kappa$ B* promoter activity and a concomitant reduction of proinflammatory cytokines expression (Fig. 4e,f and Supplementary Fig. 4e), the terminal  $\beta$ -galactosidase moiety of FetA seems crucial for recognizing TLR4. We show that  $\beta$ -galactosidase-cleaved FetA did not produce FFA-induced insulin resistance in adipocytes (Fig. 4g).

The pathway in which FFA–FetA-induced TLR4 activation causes insulin resistance is presented in a schematic diagram (Fig. 4h). The influence of dietary lipids on the activation of the TLR4–NF- $\kappa$ B pathway to produce proinflammatory cytokines, resulting in insulin resistance, has long been known. The amount of fat in HFD could be a factor in TLR4-mediated insulin resistance; 45% fat in HFD has been shown to be insufficient to produce such response<sup>35</sup>, whereas 55–60% fat in HFD effected insulin resistance through TLR4 (refs. 4,20,36). We have used HFD with 65% fat, which clearly induced insulin resistance. However, the molecular pathways that underlie a FFA-TLR4 interaction continue to elude investigators. FetA is a major carrier protein of FFAs in the circulation<sup>22</sup>, and in the absence of FFAs directly binding to TLR4<sup>4,9</sup>, we envisioned an endogenous presenter of FFAs to TLR4. We have shown here that FetA serves this purpose, as it physically interacts with both FFAs and TLR4. Moreover, our work has indicated a key role of FetA in FFA-induced TLR4 activation in adipocytes and thus in insulin resistance. These findings further suggest that FetA represents a potentially new target for developing therapeutics in the management of lipid-induced insulin resistance and type 2 diabetes.

## METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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## AUTHOR CONTRIBUTIONS

D.P. and S.D. designed and performed all the experiments, analyzed the data and wrote the manuscript. R.K. generated VMO-based FetA and *Tlr4* knockdown mice; G.D. performed lipid infusion study in WT and FetA<sup>KD</sup> mice; S.R. provided nondiabetic and diabetic human blood and fat tissue; S.S.M. provided blood and tissue samples from WT and *db/db* mice; S. Maitra, G.D., S. Mukhopadhyay and S.S.M. wrote the manuscript; S.B. designed and supervised this study, analyzed the data and wrote the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# Ultraviolet radiation damages self noncoding RNA and is detected by TLR3

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Exposure to ultraviolet B (UVB) radiation from the sun can result in sunburn, premature aging and carcinogenesis, but the mechanism responsible for acute inflammation of the skin is not well understood. Here we show that RNA is released from keratinocytes after UVB exposure and that this stimulates production of the inflammatory cytokines tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6) from nonirradiated keratinocytes and peripheral blood mononuclear cells (PBMCs). Whole-transcriptome sequencing revealed that UVB irradiation of keratinocytes induced alterations in the double-stranded domains of some noncoding RNAs. We found that this UVB-damaged RNA was sufficient to induce cytokine production from nonirradiated cells, as UVB irradiation of a purified noncoding RNA (U1 RNA) reproduced the same response as the one we observed to UVB-damaged keratinocytes. The responses to both UVB-damaged self-RNAs and UVB-damaged keratinocytes were dependent on Toll-like receptor 3 (TLR3) and Toll-like receptor adaptor molecule 1 (TRIF). In response to UVB exposure, *Tlr3*<sup>-/-</sup> mice did not upregulate TNF- $\alpha$  in the skin. Moreover, TLR3 was also necessary for UVB-radiation-induced immune suppression. These findings establish that UVB damage is detected by TLR3 and that self-RNA is a damage-associated molecular pattern that serves as an endogenous signal of solar injury.

Excess exposure to solar radiation in the UVB wavelength range (280–320 nm) is a key risk factor for skin cancer<sup>1,2</sup> and results in an inflammatory reaction of the skin commonly known as sunburn. However, despite the morbidity resulting from inflammation caused by UVB radiation, the molecular mechanisms responsible for detecting UVB injury are not completely understood. Once UVB damage occurs, several downstream responses have been described, including changes in *cis*-uroic acid (*cis*-UCA), DNA and lipids<sup>3–5</sup>. UVB exposure also triggers the activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B)<sup>6,7</sup> and the induction of cytokines, including IL-6 and TNF- $\alpha$ <sup>8</sup>. TNF- $\alpha$  is thought to have a role in the response to UVB damage, as it induces apoptosis<sup>9</sup>, is proinflammatory<sup>10,11</sup> and may mediate UVB

immunosuppression<sup>12,13</sup>, and TNF- $\alpha$ -specific antibodies reduce the number of sunburn cells after UVB exposure<sup>14</sup>. Because of the major pleiotropic, downstream effects of TNF- $\alpha$  after solar injury, we sought here to determine what elements induce the expression of this cytokine after exposure to UVB radiation.

We first determined if exposing cells to UVB radiation produced a molecule(s) capable of inducing the release of TNF- $\alpha$  from nonirradiated cells. We exposed normal human epidermal keratinocytes (NHEKs) to UVB radiation (15 mJ cm<sup>-2</sup>) and added the lysates from the UVB-irradiated keratinocytes or equal numbers of lysed nonirradiated cells to nonirradiated NHEKs and PBMCs. Lysates from UVB-irradiated NHEKs stimulated the production of TNF- $\alpha$  and IL-6 in nonirradiated, responding NHEKs and PBMCs, but lysates from nonirradiated NHEKs did not (Fig. 1a–c). The concentrations of TNF- $\alpha$  protein remained elevated at up to 48 h after exposure to UVB-irradiated keratinocyte lysates (Supplementary Fig. 1). RNase inhibited the capacity of the lysates from UVB-irradiated NHEKs to induce the production of TNF- $\alpha$  and IL-6 (Fig. 1a–c). We found that the lysates from UVB-irradiated NHEKs also induced an increase in the expression of TLR3 in responding nonirradiated NHEKs (Fig. 1d), a pattern recognition molecule that is best known for its capacity to detect dsRNA<sup>15</sup>. Targeted knockdown of TLR3 in nonirradiated responding NHEKs significantly ( $P < 0.001$ ) reduced the capacity of lysates from UVB-irradiated NHEKs to induce the production of TNF- $\alpha$  and IL-6 (Fig. 1e,f). We were also able to reproduce these events in mice, as intradermal injection of lysates from UVB-irradiated NHEKs, but not of nonirradiated NHEKs, induced redness, swelling and TNF- $\alpha$  and IL-6 cytokine production in the skin of wild-type mice but not the skin of *Tlr3*<sup>-/-</sup> mice (Fig. 1g–i and Supplementary Fig. 2). Thus, these observations suggest that recognition of UVB-induced damage may be mediated by RNA and detected by TLR3.

To identify specific alterations in RNA induced by UVB exposure that may be responsible for triggering the inflammatory response, we applied an unbiased approach using an adaptation of next-generation whole-transcriptome shotgun sequencing (RNA-Seq). This method of RNA sequencing can be dependent on the accessibility

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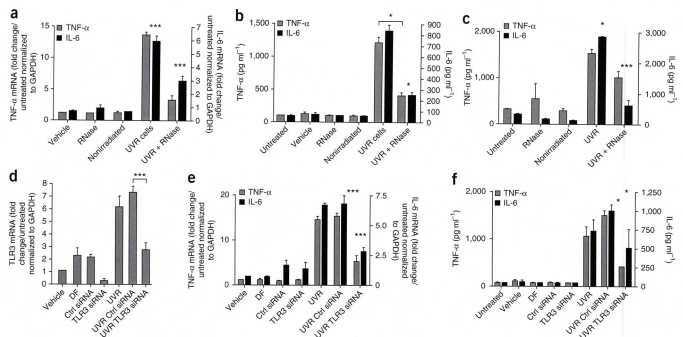


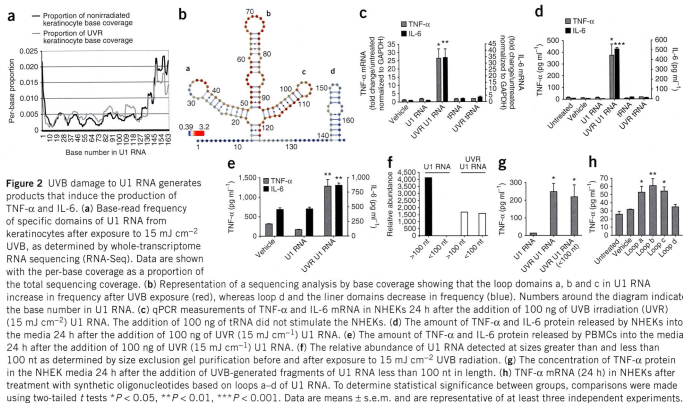
**Figure 1** RNA from UVB-irradiated keratinocytes induces the production of inflammatory cytokines. **(a)** Quantitative PCR (qPCR) measurements of TNF- $\alpha$  and IL-6 mRNA (24 h) in human keratinocytes cultured for 24 h with control NHEK lysates (nonirradiated), lysates from UVB-irradiated (UVR) NHEKs or lysates from UVR NHEKs first treated with RNase. **(b)** The concentrations of TNF- $\alpha$  and IL-6 protein measured by ELISA from culture supernatants of NHEKs after the additions described in **a**. **(c)** The concentrations of TNF- $\alpha$  and IL-6 protein from PBMCs treated with lysates from UVR NHEKs as described in **a**. **(d,e)** qPCR measurements of TLR3 mRNA (24 h) **(d)** and of TNF- $\alpha$  and IL-6 mRNA **(e)** from NHEKs treated with TLR3 siRNA or control NHEKs treated with vehicle, transfection reagent (DF) or control oligonucleotides (ctrl siRNA) and then stimulated with lysates from UVR NHEKs. **(f)** Concentrations of TNF- $\alpha$  and IL-6 protein from culture supernatant of NHEKs measured by ELISA after the experiments described in **e**. **(g)** The ears of a wild-type C57BL/6 mouse and a *Trif*<sup>-/-</sup> mouse 24 h after intradermal ear injection of lysates from UVR NHEKs or equal amounts of lysates from nonirradiated NHEKs. **(h)** Thickness of the ear skin in mice treated as described in **(g)**. **(i)** qPCR measurements of TNF- $\alpha$  and IL-6 mRNA in tissue extracts of ear skin treated as described in **(g)**. To determine statistical significance between groups, comparisons were made using two-tailed *t* tests \**P* < 0.05, \*\*\**P* < 0.001. Data are means  $\pm$  s.e.m. and are representative of at least three independent experiments. *n* = 4–6 mice per group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

of the RNA secondary structure, and a change in RNA structure induced by UVB radiation can therefore be detected by a change in the frequency of sequence reads. A comparison of the base-reading frequency obtained using RNA from lysates from UVB-irradiated NHEKs and that obtained using RNA from identical unexposed cells revealed changes in the specific domains of several small nuclear RNAs (snRNAs) (Supplementary Table 1). These snRNAs were of particular interest because they contain stem-loop structures that could form dsRNAs and thereby activate TLR3. We took particular note of snU1 RNA, as it was among the most abundant noncoding RNAs present in the irradiated cells (falling into the ninety-fourth percentile), and its sequence frequency increased by 19.5% after UVB exposure (Supplementary Table 1). An RNA-Seq analysis of the U1 RNA showed that UVB exposure increased the base-read frequency in the U1 loop domains (Fig. 2a,b). These loops generated were potential ligands for TLR3.

As U1 RNA had been suggested to modulate autoimmune responses and activate innate immune signaling<sup>16,17</sup>, we chose to test whether

direct UVB damage to this type of endogenous noncoding RNA could trigger an inflammatory response. We exposed pure, synthetic U1 RNA to UVB radiation and then added it to NHEKs or PBMCs. Nonirradiated U1 RNA (3,000 ng ml<sup>-1</sup>) had no detectable effect on inducing the production of TNF- $\alpha$  in either cell type (Fig. 2c–e). In contrast, low concentrations (100 ng ml<sup>-1</sup>) of U1 RNA that was exposed to UVB irradiation increased the production of TNF- $\alpha$  and IL-6 in both NHEKs and PBMCs at 24 h after exposure (Fig. 2c–e). The concentrations of TNF- $\alpha$  remained elevated up to 72 h after exposure in NHEKs (Supplementary Fig. 1).

To confirm that UVB irradiation altered the U1 RNA, we performed a direct analysis of synthetic U1 RNA before and after exposure to UVB radiation. Gel purification of the U1 RNA showed that UVB irradiation resulted in the production of small RNA fragments less than 100 bp in length (Fig. 2f). We obtained a similar result after an analysis of UVB-irradiated U1 RNA with capillary electrophoresis (data not shown). These purified fragments of U1 RNA generated by UVB irradiation potentially stimulate TNF- $\alpha$  production when added



to nonirradiated NHEKs (Fig. 2g). Furthermore, small synthetic oligonucleotides representing the U1 RNA loops a, b and c (Fig. 2b) induced TNF- $\alpha$  production when added to nonirradiated NHEKs, but oligonucleotides representing loop d did not show activity in this context (Fig. 2h). Thus, activity of the oligonucleotides correlated with the over-representation of loops a–c and the under-representation of loop d found by RNA-Seq analysis.

We next examined whether U1 RNA could activate TLR3 signaling. As observed with total RNA from UVB-damaged cells, UVB-irradiated U1 RNA significantly (*P* < 0.05) induced the expression of TLR3 mRNA in cultured nonirradiated NHEKs (Fig. 3a). Treatment of NHEKs with TLR3 siRNA attenuated the response to UVB-irradiated U1 RNA (Fig. 3b,c). Confocal microscopy and intracellular staining for TLR3 confirmed that UVB-irradiated U1 RNA interacts with

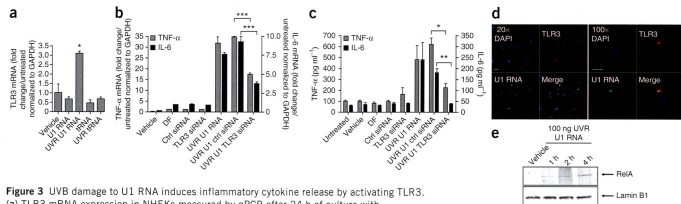
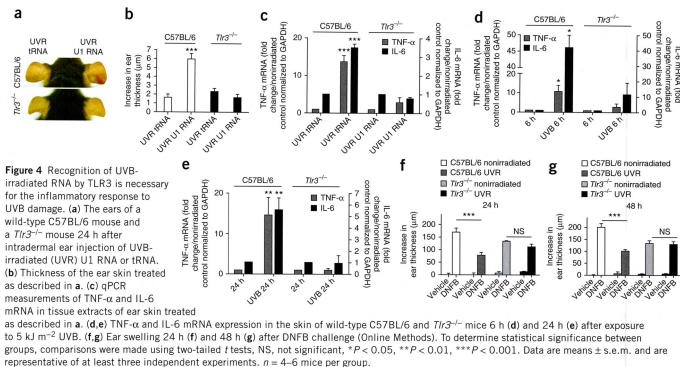


**Figure 3** UVB damage to U1 RNA induces inflammatory cytokine release by activating TLR3. (a) TLR3 mRNA expression in NHEKs measured by qPCR after 24 h of culture with UVB-irradiated (UVR) U1 RNA. (b) TNF- $\alpha$  and IL-6 mRNA expression from NHEKs treated with siRNA to TLR3 and then stimulated with UVR U1 RNA for 24 h. (c) The concentrations of TNF- $\alpha$  and IL-6 in supernatants from NHEKs treated with siRNA to TLR3. (d) Intracellular colocalization of TLR3 (red fluorescence) and UVR U1 RNA labeled with Alexa Fluor 488 (green fluorescence). The blue staining is DAPI. Scale bars, 20 $\times$  magnification, 50  $\mu$ m; 100 $\times$  magnification, 20  $\mu$ m. (e) RelA and lamin B1 (loading control) detected by western blot of nuclear lysates of NHEKs treated with UVR U1 RNA for 1, 2 or 4 h. (f) Results from NHEKs cultured to 70% confluency on chamber slides, treated with siRNA to TLR3 and exposed to UVR U1 RNA for 4 h. Cells were fixed and stained with an antibody to RelA (red). Scale bar, 50  $\mu$ m. To determine statistical significance between groups, comparisons were made using two-tailed *t* tests \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001. Data are means  $\pm$  s.e.m. and are representative of at least three independent experiments.



endosomal TLR3 in NHEKs (Fig. 3d). UVB-irradiated U1 RNA also induced the translocation of the transcription factor RelA (also known as p65) to the nucleus, as assayed by western blot (Fig. 3e), and this was dependent on TLR3, as assayed by direct immunofluorescence (Fig. 3f). Furthermore, pretreatment of NHEKs for 2 h with either dextran sulfate or fucoidin, two inhibitors of scavenger receptor function and dsRNA uptake, inhibited the TNF- $\alpha$ -inducing activity of the UVB-irradiated U1 RNA, suggesting uptake and endosomal delivery of U1 RNA is required to activate TLR3 (Supplementary Fig. 3).

Next, we assessed the effects of U1 RNA *in vivo*. UVB-irradiated U1 RNA induced redness and increased ear thickness in mouse ears, whereas transfer RNA (tRNA) exposed to UVB did not (Fig. 4a,b). UVB-irradiated U1 RNA also increased the expression of TNF- $\alpha$  and IL-6 mRNA in the skin of wild-type control but not *Trir*<sup>-/-</sup> mice (Fig. 4a–c). Furthermore, the inflammatory response to UVB-exposed U1 RNA was blunted in mice deficient in TRIF, an essential downstream element in TLR3 signaling (Supplementary Fig. 4).

To assess the role of TLR3 in the response to UVB radiation, we directly irradiated mice and measured both the inflammatory response and UVB-radiation-induced immunosuppression. Six hours and 24 h after UVB exposure, upregulation of TNF- $\alpha$  or IL-6 mRNA in the skin was reduced in *Trir*<sup>-/-</sup> mice as compared to wild-type littermates (Fig. 4d,e). In addition, UVB exposure did not induce the production of inflammatory cytokines in *Trir*<sup>-/-</sup> mice (Supplementary Fig. 4). *Trir*<sup>-/-</sup> mice also showed less skin redness compared to wild-type controls after UVB radiation exposure (Supplementary Fig. 5). Although IL-1 stimulates the production of TNF- $\alpha$  and has a key role in inflammation after UVB exposure<sup>18,19</sup>, TNF- $\alpha$  was not induced in the skin of IL-1 receptor knockout mice (*Il1r*<sup>-/-</sup>) after UVB exposure (Supplementary Fig. 6). TLR3 was also necessary for UVB-radiation-induced immunosuppression after UVB exposure. Twenty-four hours after sensitization and challenge with dinitrofluorobenzene (DNFB), nonirradiated wild-type mice developed an allergic contact hypersensitivity (CHS) reaction (Fig. 4f,g). Exposure to UVB radiation

72 h before sensitization to DNFB suppressed the CHS response in wild-type but not in *Trir*<sup>-/-</sup> mice (Fig. 4f,g and Online Methods), suggesting that TLR3 is crucial for the ability of UVB radiation to suppress the CHS response.

The recognition of RNA involves a coordinated response between TLRs and cytoplasmic RNA helicases. In our studies, however, there was little indication of any involvement of other RNA sensors. The induction of TNF- $\alpha$  and IL-6 were not altered after UVB exposure of mice deficient in mitochondrial antiviral signaling protein (MAVS), the adaptor for intracellular RNA helicases such as melanoma differentiation-associated gene 5 (MDA5) and retinoic acid inducible gene-1 (RIG-1) (Supplementary Fig. 7). Furthermore, the use of a pharmacologic inhibitor of this pathway did not block the induction of cytokines by lysates from UVB-irradiated NHEKs (Supplementary Fig. 7).

Based on these observations, we propose that noncoding RNAs damaged by UVB radiation are partially responsible for UVB-induced inflammation and act as previously unknown damage-associated molecular patterns. In support of our hypothesis are prior observations that UVB radiation can induce RNA-RNA crosslinking, pyrimidine dimer formation and the oxidation of guanine to 8-oxo-7,8-dihydroguanosine (8-oxoG)<sup>20</sup>. Other molecules may also activate TLR3 in the whole-cell system, but we here show specifically that a specific form of RNA altered by UVB irradiation can activate TLR3, as isolated tRNA and nonirradiated U1 RNA were inactive in this context. In addition, other products generated after UV exposure, such as *cis*-UCA<sup>21</sup>, telomeric nucleotides<sup>22,23</sup>, 6-formylindolo(3,2-*b*) carbazole<sup>24</sup> and IL-1 (refs. 25,26), may also contribute to UVB-induced inflammation but probably do not contribute substantially to the immediate production of TNF- $\alpha$  and IL-6, as this response was dependent on TLR3.

These observations provide a new framework to understand the UVB damage response and may influence the understanding of a wide range of photosensitive phenomena. They suggest the potential

for RNAs as biomarkers of UVB-induced injury, the use of RNAs as alternatives to phototherapy and the potential for blocking damaged self-RNA recognition to be used as a therapeutic target for photo-sensitive disorders. The TLR3-mediated cytokine response may have major implications for tumorigenesis because of the many associations of TNF- $\alpha$  with apoptosis, inflammation and immune surveillance. Future work to better define the role of TLR3 in the outcomes occurring after UVB exposure is therefore warranted.

## METHODS

Methods and any associated references are available in the online version of the paper.

*Note: Supplementary information is available in the online version of the paper.*

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## AUTHOR CONTRIBUTIONS

J.B. performed most of the experiments, analyzed results and wrote the manuscript. T.N., J.M., B.M. and A.W.B. assisted with mouse experiments and reviewed the manuscript. C.C.-Z. and B.D.Y. analyzed RNA-Seq results and reviewed the manuscript. E.L.G. and L.M. provided reagents, helped with the design and interpretation of experiments involving U1 RNA and reviewed the manuscript. R.L.G. supervised and designed experiments and wrote and prepared the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# Large intestine–targeted, nanoparticle-releasing oral vaccine to control genitorectal viral infection

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Both rectal and vaginal mucosal surfaces serve as transmission routes for pathogenic microorganisms. Vaccination through large intestinal mucosa, previously proven protective for both of these mucosal sites in animal studies, can be achieved successfully by direct intracolorectal (i.c.r.) administration, but this route is clinically impractical. Oral vaccine delivery seems preferable but runs the risk of the vaccine's destruction in the upper gastrointestinal tract. Therefore, we designed a large intestine–targeted oral delivery with pH-dependent microparticles containing vaccine nanoparticles, which induced colorectal immunity in mice comparably to colorectal vaccination and protected against rectal and vaginal viral challenge. Conversely, vaccine targeted to the small intestine induced only small intestinal immunity and provided no rectal or vaginal protection, demonstrating functional compartmentalization within the gut mucosal immune system. Therefore, using this oral vaccine delivery system to target the large intestine, but not the small intestine, may represent a feasible new strategy for immune protection of rectal and vaginal mucosa.

Mucosal immunization has proven to be crucial for inducing mucosal protection<sup>1–5</sup> and contributes to rapid and long-lasting mucosal protection, in contrast to systemic immunization<sup>6</sup>. It has been shown that antigen-specific functional CD8<sup>+</sup> cytotoxic T cells in the mucosa are essential for protection of rhesus macaques from CD4<sup>+</sup> T cell depletion by simian-human immunodeficiency virus<sup>3</sup>, whereas human studies indicate that a higher frequency of the antigen-specific mucosal CD8<sup>+</sup> T cells correlates with a lower degree of herpes simplex viral infectivity as well as reduced severity of the disease<sup>7</sup>. In the mucosal tissues of HIV-infected long-term nonprogressors, there exist immunodominant CD8<sup>+</sup> T cells, and their presence is strongly correlated with HIV-1 control<sup>5,8</sup>. A variety of approaches have been proposed and employed to induce protective mucosal

immunity against viral transmission through either the rectal or vaginal route<sup>1–3,9–11</sup>. However, a potent but clinically practical genitorectal vaccination strategy remains unestablished for several reasons. The large intestinal mucosa is an optimal site to induce both rectal and vaginal immunity. I.c.r. vaccination directly at the large intestinal mucosa induces robust cellular and humoral immune responses in the regional lymph nodes<sup>4</sup> more effectively than vaccination at a distant mucosa (for example, intranasal) or by a parenteral route<sup>1–5</sup>. However, for mass human vaccination, i.c.r. administration seems to be clinically too cumbersome and unpalatable. In addition, this procedure, although generally low risk, has the potential to be physically traumatic. The intranasal route, although practical and relatively easy, poses the risk of inoculum invasion into the central nervous system by olfactory nerve transport<sup>12</sup>. The oral route is the safest and most practical; however, except for a few live attenuated vaccines inducing systemic responses, simple oral delivery is ineffective at protecting either rectal or genital mucosa<sup>13</sup>. The failure is mostly attributed to pH-dependent or enzymatic destruction of the vaccine in the proximal gut and, probably, inadequate antigen uptake in the large intestine.

We aimed here to discover a way to selectively deliver a vaccine to the large intestinal mucosa through the oral route, which to our knowledge has not previously been accomplished. To mimic the 'gold standard' i.c.r. immunization while circumventing the limitations of oral delivery, we encapsulated a peptide or protein vaccine into biologically compatible poly(DL-lactic-co-glycolic acid) (PLGA) nanoparticles<sup>14,15</sup> to be used for site-specific immunization. The depot effect of PLGA nanoparticles offers an additional feature in that its controlled release of entrapped vaccines over extended time periods provides a longer antigen exposure to the immune system. PLGA particle size, adjustable during manufacturing, was engineered in nanometers because size-dependent mucosal uptake is most effective within nanometer ranges and impeded when the size is over 1  $\mu\text{m}$  (ref. 16). Selective combinations of Toll-like receptor (TLR)

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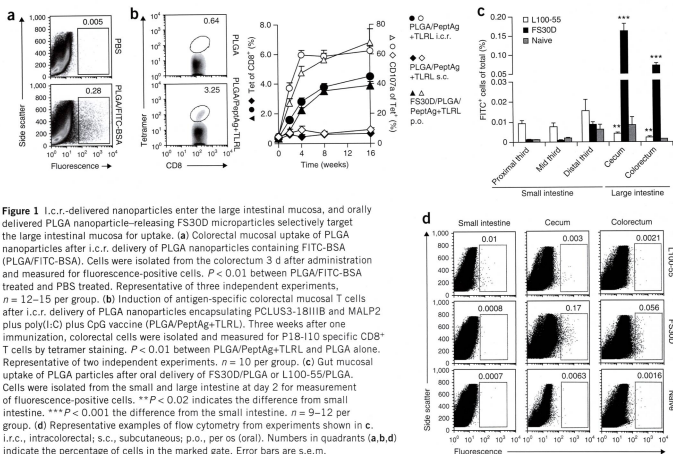


**Figure 1** I.c.r.-delivered nanoparticles enter the large intestinal mucosa, and orally delivered PLGA nanoparticle-releasing FS30D microparticles selectively target the large intestinal mucosa for uptake. **(a)** Colocal mucosal uptake of PLGA nanoparticles after i.c.r. delivery of PLGA nanoparticles containing FITC-BSA (PLGA/FITC-BSA). Cells were isolated from the colorectum 3 d after administration and measured for fluorescence-positive cells.  $P < 0.01$  between PLGA/FITC-BSA treated and PBS treated. Representative of three independent experiments,  $n = 12$ –15 per group. **(b)** Induction of antigen-specific colorectal mucosal T cells after i.c.r. delivery of PLGA nanoparticles encapsulating PCLUS3-18IIIB and MALP2 plus poly(I:C) plus CpG vaccine (PLGA/PeptAg+TLRL). Three weeks after one immunization, colorectal cells were isolated and measured for P18-I10 specific CD8<sup>+</sup> T cells by tetramer staining.  $P < 0.01$  between PLGA/PeptAg+TLRL and PLGA alone. Representative of two independent experiments,  $n = 10$  per group. **(c)** Gut mucosal uptake of PLGA particles after oral delivery of FS30D/PLGA or L100-55/PLGA. Cells were isolated from the small and large intestine at day 2 for measurement of fluorescence-positive cells.  $**P < 0.02$  indicates the difference from small intestine.  $***P < 0.001$  the difference from the small intestine.  $n = 9$ –12 per group. **(d)** Representative examples of flow cytometry from experiments shown in c. I.c.r., intracolorectal; s.c., subcutaneous; p.o., per os (oral). Numbers in quadrants (**a**, **b**, **d**) indicate the percentage of cells in the marked gate. Error bars are s.e.m.

ligands can induce synergistic activation of T cells<sup>17–19</sup>. As vaccine adjuvants, we used macrophage-activating lipoprotein (MALP-2) for TLR2, polyinosine-polycytidylic acid (poly(I:C)) for TLR3 and CpG oligodeoxynucleotides (ODN) for TLR9, which have been shown to synergistically induce mucosal antiviral protection after i.c.r. immunization<sup>20</sup>.

To bypass the denaturing effects of digestive low pH and enzymatic destruction and to selectively deliver the particles to the lower gastrointestinal tract intact, we coated the PLGA nanoparticle surface with methacrylate-based polymer Eudragit FS30D<sup>21</sup>, an anionic tripolymer comprising poly(methyl acrylate, methyl methacrylate, methacrylic acid) in a 7:3:1 ratio. The ratio of free carboxyl groups to ester groups is ~1:10. It is pH sensitive and soluble in intestinal fluids at pH > 7.0, seen only in the terminal ileum, thereby preventing the contents from being released too early. These coated particles are  $\geq 10 \mu\text{m}$  in diameter to avoid premature uptake in the small intestine primarily by Peyer's patches, which are effective at taking up particles up to  $1 \mu\text{m}$  (ref. 16). Our design is shown in **Supplementary Figure 1**.

## RESULTS

### Proof-of-principle study on the PLGA nanoparticle vaccine

Uncoated PLGA nanoparticles were manufactured in the range of 300–500 nm ( $418 \pm 88 \text{ nm}$ ) (mean  $\pm$  s.d.) in diameter (**Supplementary Fig. 2a**), with 90% encapsulation efficacy (**Supplementary Fig. 2b**). We first conducted a proof-of-principle experiment in which fluorescein-conjugated BSA formulated in PLGA (PLGA/FITC-BSA) nanoparticles were delivered directly to the colon of mice by i.c.r. administration. At day 3, a proportion of the cells isolated

from the large intestinal lamina propria were detected positive for fluorescence (**Fig. 1a**), indicating an uptake of the nanoparticles. Nanoparticle uptake was primarily found in CD11b<sup>+</sup>B220<sup>int</sup> macrophages and secondarily in CD11c<sup>+</sup>CD11b<sup>+</sup> dendritic cells (DCs) in the lamina propria of the large intestine (**Supplementary Fig. 3a**). Transmission electron microscopy showed PLGA nanoparticles in the cytoplasm (**Supplementary Fig. 3b**). Uptake of PLGA nanoparticles by DCs (derived from bone marrow) was also confirmed *in vitro* by flow cytometry (**Supplementary Fig. 4a**), fluorescence microscopy (**Supplementary Fig. 4b**) and transmission electron microscopy (**Supplementary Fig. 4c**).

We subsequently confirmed that antigen-specific T cell responses could be induced in the colon after a single i.c.r. delivery (**Fig. 1b**), but not after subcutaneous administration (**Supplementary Fig. 5**), of PLGA nanoparticles encapsulating PCLUS3-18IIIB (a CD4<sup>+</sup> T cell helper epitope fused with an HIV Env CD8<sup>+</sup> cytotoxic T lymphocyte epitope) and TLR ligands (MALP2 plus poly(I:C) plus CpG)<sup>20</sup>. Therefore, PLGA nanoparticles can serve as an effective vaccine delivery system when they are deposited in the large intestinal lumen.

### Oral delivery of nanoparticle-releasing microparticle vaccines

The Eudragit FS30D polymer served to make 10- to 50- $\mu\text{m}$  particles, too large to be phagocytosed, that released a substantial amount of their contents as quickly as 1 h at pH 7.4, in contrast to at pH 2.5, at which the particles were stable (**Supplementary Fig. 6**). After oral delivery of Eudragit FS30D containing PLGA/FITC-BSA nanoparticles, nanoparticle uptake occurred almost exclusively in the large

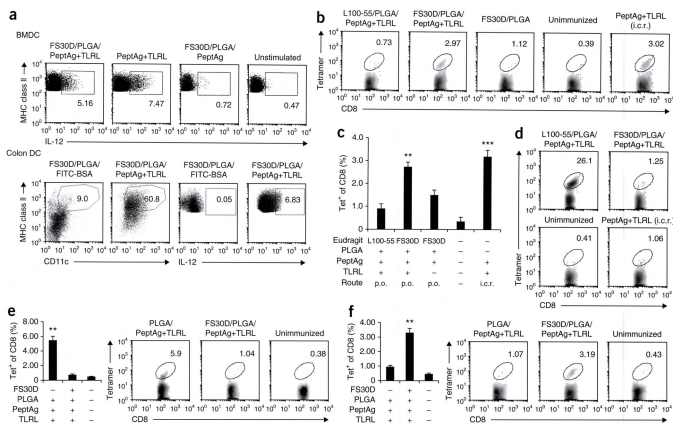


**Figure 2** Orally delivered, FS30D-coated PLGA nanoparticle vaccine induces antigen-specific T cells in the large intestine, whereas L100-55-coated vaccine induces T cells in the small intestine. **(a)** Activation of DCs after a 20-h incubation with supernatants from FS30D-coated PLGA containing PCLUS3-18IIIB plus MALP2, poly(I:C) and CpG (FS30D/PLGA/PeptAg+TLRL), antigen (FS30D/PLGA/PeptAg) or vaccine only (PeptAg+TLRL) dissolved in PBS at pH 7.4 for 16 h (top graphs). Intracellular interleukin-12 (IL-12) was measured by flow cytometry ( $n = 6$  per group). The nanoparticle-releasing microparticles were also given orally, and DC activation *in vivo* was assessed *ex vivo* (bottom graphs),  $n = 5$  per group. MHC, major histocompatibility complex. **(b–d)** Induction of T cell responses after oral delivery of FS30D/PLGA/PeptAg+TLRL or L100-55/PLGA/PeptAg+TLRL. Oral administration was conducted twice with a 2-week interval. Tetramer-positive cells in the colorectum (**b** and **c**) or upper part of the small intestine (**d**). Oral administration was conducted three weeks later. The i.c.r. group was immunized with vaccine only without nanoparticles but formulated in DOTAP.  $^{**}P < 0.01$ ,  $^{***}P < 0.001$  indicate the significant difference between the group with asterisks and each of the groups without asterisks. There are no differences between the two groups with asterisks. A representative experiment on tetramer results in the colorectum is shown in **b**, and all such experiments are summarized with statistics ( $n = 8–12$  per group) in **c**. In **d**,  $P < 0.001$  for L100-55 versus other groups.  $n = 8–12$  per group. **(e,f)** T cell responses induced after oral delivery of uncoated or FS30D-coated PLGA/PeptAg+TLRL. Tetramer-positive cells in the upper small intestine (**e**) and the colorectum (**f**) were measured.  $^{**}P < 0.01$  indicates a significant difference between groups ( $n = 7$  per group). Numbers in quadrants (**a,b,d–f**) indicate the percentage of cells in the marked gate. Error bars are s.e.m.

intestine (Fig. 1c,d). The cecum is the first part of the large intestine encountered by the particles, but in humans, where the cecum is relatively small, the starting point of nanoparticle release and selective uptake in the large intestine may differ somewhat. We further contrasted Eudragit FS30D with another Eudragit formulation, L100-55, that starts to dissolve at pH  $> 5.5$ , allowing early release. We found that oral administration of L100-55-coated nanoparticles resulted in primary uptake in the small intestine (Fig. 1c,d). Likewise, uncoated PLGA nanoparticles, to the extent that any successfully traversed the stomach, were primarily delivered to the small intestine and not the colon (Supplementary Fig. 7). Thus, the distribution of nanoparticles was altered by coating them with FS30D, such that a majority reached and were taken up by cells in the large intestine (Fig. 1c,d and Supplementary Fig. 7). These results validate the approach of coating PLGA nanoparticles with FS30D to protect them during transit through the upper gastrointestinal tract for improved delivery to the large intestine.

We next used a luciferase reporter system to confirm the large intestine-targeted delivery with FS30D. We detected luciferase

expression specifically in large intestinal sections after oral administration of FS30D-coated PLGA nanoparticles containing a luciferase DNA plasmid (Supplementary Fig. 8).

After confirming that formulated vaccine components within the nanoparticle-releasing microparticles retained TLR agonist activity *in vitro* settings or after oral administration (Fig. 2a), we examined intestinal immune responses in mice after two immunizations with the two different Eudragit coatings of the PLGA nanoparticle vaccine given orally with a 2-week interval. Three weeks after the second immunization with the oral FS30D-coated vaccine, we detected tetramer-positive CD8<sup>+</sup> T cells in the large intestine, indicating successful induction of colorectal immunity (Fig. 2b,c). In contrast, the L100-55-coated vaccine induced a minimal number of colonic antigen-specific CD8<sup>+</sup> T cells (Fig. 2b,c). In fact, the L100-55-coated vaccine induced a T cell response primarily in the small intestine, where the FS30D-coated vaccine was only marginally effective (Fig. 2d). Without Eudragit coating, PLGA nanoparticle vaccines did not elicit significant immune responses in the large intestine but did induce responses in the small intestine (Fig. 2e,f). These results

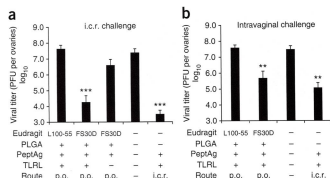
**Figure 3** Orally delivered FS30D-coated PLGA nanoparticle peptide vaccine confers T cell-mediated resistance to virus infection at the rectal or vaginal mucosa. **(a,b)** Viral load in ovaries on a log scale after FS30D/PLGA/ProtAg+TLR or L100-55/PLGA/ProtAg+TLRL was given orally to mice twice with a 2-week interval, followed by i.c.r. **(a)** or intravaginal **(b)** challenge with  $2 \times 10^7$  or  $1 \times 10^7$  PFU of vPE16, respectively, 3 weeks after the last immunization. Ovaries (where this virus primarily replicates) were removed at day 6 for viral titer assessment.  $^{**}P < 0.01$ ,  $^{***}P < 0.001$  indicate the significant difference in viral titer between the group with asterisks and each of the groups without asterisks ( $n = 12$ –15 per group). PFU, plaque-forming units. Error bars are s.e.m.

affirm that the enteric coating with FS30D is essential for oral delivery of PLGA nanoparticles to the large intestine.

# Induction of T cell immunity against viral infection

To evaluate the protective efficacy of this vaccine, after prime and boost oral immunization we challenged mice rectally with a replication-competent vaccinia virus, vPE-16, which expresses the HIV Env protein containing the epitope P18-I10 used in the peptide vaccine. Mice immunized with the L100-55-coated vaccine were not protected from the virus challenge. However, the FS30D-coated vaccine reduced viral load almost equally as well as the peptide vaccine given i.c.r. (Fig. 3a). Therefore, oral delivery of FS30D-coated PLGA vaccine targeting the large intestine is effective for local colorectal vaccination against viral infection.

It has been previously shown that i.c.r. vaccination with adenovirus-based vaccines induces effective protection against virus challenge (vaccinia and herpes simplex virus-2) not only in the rectal but also in the vaginal mucosa<sup>4</sup>. To determine whether vaginal mucosal protection can be induced by the orally administered FS30D-coated PLGA vaccine, we immunized mice orally with the vaccine (FS30D-coated PLGA containing PLUS3-18IIB plus TLR ligands) twice with a 2-week interval, followed by intravaginal challenge with vPE-16. Compared to i.c.r. immunization, oral delivery of FS30D-coated vaccine induced almost equal clearance of virus after vaginal challenge (Fig. 3b), whereas, again, the L100-55 formulation that delivers the vaccine to the small intestine was not effective. Thus, delivery to the large intestine was more effective than to the small intestine in protecting against both genital and rectal challenge. This efficacy was largely T-cell mediated, as



the virus does not incorporate the HIV-1 Env protein gp160 into virions and is not sensitive to gp160 neutralization.

# Induction of antibody immunity against viral infection

The humoral response also has a key role in protection in the gut and in genital mucosal immunity. Therefore, we examined whether encapsulating whole viral proteins in the FS30D-coated PLGA vaccine can induce antibody-mediated protective immunity at both mucosal sites after challenging mice with the pathogenic vaccinia strain WR. Vaccinia A33 and L1 are immunogens of the extracellular enveloped virion and intracellular mature virion, respectively. Antibody responses induced by the combination of both types of viral antigens encoded by plasmid DNA<sup>22</sup> or as recombinant proteins<sup>23</sup> can protect animals from lethal challenge by WR. Of note, cytotoxic T lymphocytes specific for the vaccinia protein A33 or L1 have not been reported in BALB/c mice<sup>24</sup>. TLR ligands have been shown to activate B cells directly and contribute to antigen-specific antibody responses<sup>25,26</sup>, including in the gut<sup>27</sup>. Here we found that the triple combination of MALP-2, poly(I:C) and CpG ODN TLR ligands previously shown to synergistically activate naive T cells<sup>20</sup> could also synergistically activate B cells (Supplementary Fig. 9a), as determined by CD69 upregulation<sup>28</sup>. I.c.r. immunization with the combination of recombinant A33 and L1 mixed with the triple combination of MALP-2, poly(I:C) and CpG ODN TLR ligands in DOTAP transfection reagent induced antibody responses in the blood (Supplementary Fig. 9b).

The ability of PLGA nanoparticles containing the above vaccine components to induce antibody responses was assessed by immunization through either the i.c.r. or subcutaneous routes (Supplementary Fig. 10a), and the results further showed that CD4<sup>+</sup> T cells were also activated by such a vaccine regimen (Supplementary Fig. 10b). We therefore constructed vaccine microparticles of FS30D-coated PLGA nanoparticles containing A33 protein plus L1 protein plus MALP-2,

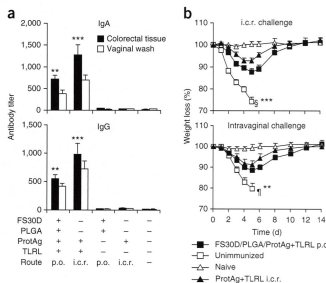


**Figure 4** Orally delivered FS30D-coated PLGA nanoparticle protein vaccine confers antibody-mediated resistance to virus infection in the rectal or vaginal tract. FS30D-coated PLGA containing antigen proteins A33, and L1 and TLR ligands (ProtAg+TLRL) was administered orally with a 2-week interval. **(a)** Local IgA (top) and IgG (bottom) antibodies against both A33 and L1 (together) measured at 3 weeks after the last immunization. Both FS30D/PLGA/ProtAg+TLRL p.o. and ProtAg+TLRL i.c.r. groups have significantly higher antibody titers than the other groups ( $P < 0.02$ ).  $^{**}P < 0.01$  and  $^{***}P < 0.001$  indicate difference (for both sites) from each of the bars without asterisks. Results from two independent experiments ( $n = 10$  per group). **(b)** Disease course of the mice assessed by weight loss after challenge with WR by the i.c.r. ( $4 \times 10^7$  PFU) or intravaginal ( $1 \times 10^7$  PFU) route 3 weeks after the last immunization.  $^{**}P < 0.02$ ,  $^{***}P < 0.001$  indicate the differences between the FS30D/PLGA/ProtAg+TLRL and unimmunized groups in weight loss ( $n = 10$ –14 per group). §, 75% mortality; ¶, 50% mortality. Error bars are s.e.m.

poly(I:C) and CpG ODN TLR ligands and administered them orally. Vaccinia-specific IgA and IgG antibody responses were significantly induced in both the large intestine (tested in tissue homogenates) and vaginal tracts (tested in vaginal washes) (Fig. 4a). Further, orally immunized mice resisted the WR virus challenge by either the rectal or vaginal route (Fig. 4b), as determined by weight loss and by 0% mortality for immunized mice challenged by either route compared to 75% mortality for i.c.r. challenge without preimmunization and 50% mortality for intravaginal challenge without preimmunization). Therefore, the FS30D-coated PLGA vaccine system can also be used to induce antibody-mediated mucosal protection at both mucosal transmission sites.

## DISCUSSION

As direct rectal or vaginal vaccine delivery may be impractical for mass human vaccination, despite the efficacy of the i.c.r. route, oral vaccination is a desirable alternative, potentially allowing for safe, simple and rapid delivery. We hypothesized that if the vaccine is able to survive the passage to and is not absorbed before reaching the large intestine, the vaccination should mimic the efficacy of i.c.r. vaccination. Accordingly, we designed a two-part PLGA nanoparticle-releasing FS30D microparticle system and showed that this new system can bypass the small intestine and deliver orally administered vaccines highly specifically into the large intestinal mucosa and induce almost equal protective immunity in not only the rectal but also the vaginal mucosa.

Uncoated PLGA particles given orally either failed to traverse the stomach or reached only the small intestine and did not elicit significant colonic responses, only small intestinal immune responses. These results underline the need for combinatorial use of Eudragit FS30D and PLGA to ensure specific targeting of an orally delivered vaccine to the large intestine. Our study also indicates that activities of MALP-2, poly(I:C), and CpG ODN TLR ligands were maintained after encapsulation within PLGA; thus, PLGA is an ideal delivery vehicle for vaccines containing TLR agonists.

Further, we designed a contrasting coated nanoparticle (L100-55-coated PLGA nanoparticle) vaccine that delivered antigen to and induced T cell responses in the small intestine selectively, providing a way to compare these small- and large-intestinal mucosal sites for the first time with the same vaccine. Thus, we have made a fundamental discovery of cellular immune compartmentalization within the gut mucosal immune system, which is reminiscent of the subcompartmentalized gut humoral immunity<sup>29</sup>, highlighting the need for site-specific delivery of vaccines to the large intestinal mucosa for protection against infections transmitted by the rectal or vaginal route, and we have demonstrated a new way to accomplish this via the more practical oral route. Our findings thus strengthen the conception that the common mucosal immune system is somehow subcompartmentalized.

There are many potential applications of our delivery technology beyond vaccination against viral infection. This strategy is applicable for many forms of vaccines such as DNA, recombinant proteins, peptides and a wide variety of adjuvants, and it may be adapted in the development of vaccination strategies combating certain sexually transmitted infections caused by not only viruses but possibly also other types of pathogens. It also suggests a new approach to the development of a preventive or therapeutic vaccination against mucosal malignancies such as colorectal as well as cervical cancer. With this technology, in-depth study of the mucosal immunological subcompartmentalization between the large and small intestines

can be easily conducted. For example, aside from vaccination, one could use this approach to localize the site of induction of oral tolerance. The technology may be incorporated with other technologies to devise therapies in which selective targeting within the gut mucosa is needed.

Our targeted nanoparticle-releasing microparticle system lends itself to practical large-scale clinical applications because of its great stability in a dry-powder formulation, easy shipment and storage without refrigeration and long shelf life, and economical large-batch good manufacturing process processing. These features would be highly desired for effective industrial manufacturing and clinical management. PLGA is a component in several US Food and Drug Administration-approved products. Although fine tuning of the formulation may be necessary to account for longer transit times in the gut of nonhuman primates and humans, as well as slightly higher pH values (around 7.3) in the distal small intestine of humans, this study is a promising proof of concept that this type of formulation may be practical and effective for oral vaccination to induce lower gastrointestinal immunity without the need for intrarectal delivery. Capsules either coated with or composed of Eudragit have been investigated in humans for site-targeted release in the distal gastrointestinal tract<sup>30-32</sup>. Clinical approaches can be extended to consider packaging PLGA nanoparticles within these colon-specific capsules, which may represent a preferable carrier for targeted colon vaccination in humans.

In conclusion, we have demonstrated functional immune compartmentalization of the gut mucosa and developed a nanoparticle-releasing microparticle oral delivery system and demonstrated that it is an easy, noninvasive vaccination strategy effective against viral infection occurring through the rectal or vaginal mucosa. Such a vaccination strategy would represent a conceptually novel approach to the development of vaccines against mucosal infections and potentially against mucosal cancers and to the advanced study of immunobiological mechanisms involving mucosal compartmentalization.

## METHODS

Methods and any associated references are available in the online version of the paper.

*Note: Supplementary information is available in the online version of the paper.*

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## AUTHOR CONTRIBUTIONS

Q.Z., R.J.M. and J.A.B. designed the experiments, interpreted the data and wrote the manuscript. Q.Z. executed many of the experiments. Z.W. conducted some of the experiments and J.T., R.C.W., J.K. and B.E. produced nanoparticle-releasing microparticle vaccines and were involved in micro- and nanoparticle characterization and *in vitro* release experiments. T.C. participated in experiment design and the initial experiments. G.Z. performed electron microscopy. D.M.K. provided CpG ODN and contributed to analysis and discussion. I.M.B., S.G. and Y.S. participated in planning and discussion. J.A.B. oversaw the overall execution of the projects.



## COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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# Simultaneous functional photoacoustic and ultrasonic endoscopy of internal organs *in vivo*

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**At present, clinicians routinely apply ultrasound endoscopy in a variety of interventional procedures that provide treatment solutions for diseased organs. Ultrasound endoscopy not only produces high-resolution images, but also is safe for clinical use and broadly applicable. However, for soft tissue imaging, its mechanical wave-based image contrast fundamentally limits its ability to provide physiologically specific functional information. By contrast, photoacoustic endoscopy possesses a unique combination of functional optical contrast and high spatial resolution at clinically relevant depths, ideal for imaging soft tissues. With these attributes, photoacoustic endoscopy can overcome the current limitations of ultrasound endoscopy. Moreover, the benefits of photoacoustic imaging do not come at the expense of existing ultrasound functions; photoacoustic endoscopy systems are inherently compatible with ultrasound imaging, thereby enabling multimodality imaging with complementary contrast. Here we present simultaneous photoacoustic and ultrasonic dual-mode endoscopy and show its ability to image internal organs *in vivo*, thus illustrating its potential clinical application.**

Endoscopic ultrasound (EUS)<sup>1,2</sup> is currently the most clinically translated tomographic endoscopic imaging modality, and it has made key contributions to medicine by enabling new interventional procedures in many medical areas, such as gastroenterology<sup>1,3–6</sup>, pulmonology<sup>1,7,8</sup> and urology<sup>1,9,10</sup>. Representative applications<sup>1,2</sup> of this technique include EUS-guided sample collection from disease-affected tissue (so-called fine needle aspiration); tissue ablation or endosurgery; interventional device deployment or implantation; and oncologic treatments, such as fine needle injection of chemotherapeutic drugs. Compared with other mainstream medical imaging modalities, such as magnetic resonance imaging, X-ray computed tomography and positron emission tomography, ultrasound imaging is easily embodied in an endoscopic probe, uses extremely safe mechanical waves and permits bedside operation. Moreover, it provides high-speed, high-resolution, cross-sectional imaging over a large field of view (FOV).

Lung, colorectal, pancreatic and prostate cancer are leading causes of cancer mortality<sup>11</sup>, and all these cancers are located in or close to

the respiratory system or gastrointestinal tract, in which endoscopic procedures are applicable. Although EUS-based imaging studies have shown the ability to diagnose these cancers<sup>1,3–10</sup>, most of the current applications of EUS are primarily focused on guiding other interventional procedures<sup>1,2</sup>. Early-stage tumor detection or *in situ* characterization of diseased tissues is challenging for EUS because its contrast mechanism relies on bulk mechanical properties. Tumor boundaries and connections with surrounding blood or lymphatic vessels are clinically relevant and provide necessary information for assessing disease stage or progress<sup>12</sup> and planning treatment therapies. However, pure ultrasound-based image contrast does not sufficiently provide this crucial information. Recently developed optical endoscopic imaging modalities, such as endoscopic optical coherence tomography<sup>13–15</sup>, confocal endoscopy<sup>16</sup> and endoscopic polarized scanning spectroscopy<sup>17,18</sup>, can detect diseased tissues and abnormal tissue changes, such as dysplasia, with high sensitivity and high spatial resolution. Still, these techniques are limited by their inability to image targets beyond a ~1–2 mm depth because of the strong optical scattering properties of tissue.

Photoacoustic tomography (PAT) is a new imaging modality that can provide volumetric images of biological tissues *in vivo* with high spatial resolution at depths<sup>19–21</sup> that far exceed the penetration depths of conventional high-resolution optical imaging modalities<sup>13–18</sup>. PAT systems are very similar to ultrasound imaging systems because they both use ultrasound detection to render an image; however, PAT produces images with optical absorption-based contrast. By using deeply penetrable diffused light to excite photoacoustic signals, PAT can image targets several centimeters deep in soft tissues<sup>21</sup>. With high optical contrast, tumor boundaries and surrounding lymphovascular systems can be resolved with endogenous or exogenous contrast agents<sup>22–26</sup>. Additionally, other types of physiological or functional information, including total hemoglobin concentration, oxygen saturation of hemoglobin (sO<sub>2</sub>)<sup>22</sup>, blood flow<sup>27</sup> or temperature<sup>28,29</sup>, are also measurable. Therefore, photoacoustic endoscopy (PAE)<sup>30</sup> that embodies PAT in a small probe can be used as a new, minimally invasive diagnostic tool for providing optical absorption-based contrast with high spatial resolution while maintaining the capabilities and benefits of traditional EUS. Here we report what is, to our knowledge,

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the first *in vivo* demonstrations of simultaneous PAE and EUS imaging in the upper and lower gastrointestinal tracts of animals with our new integrated endoscopic system.

# RESULTS

We developed an integrated PAE and EUS imaging system for simultaneous, photoacoustic and ultrasonic imaging of internal organs *in vivo* (Fig. 1, **Supplementary Figs. 1 and 2** and **Supplementary Video 1**). The ultrasonic images are produced with conventional pulse-echo imaging that detects acoustic waves reflected from target tissue; the photoacoustic images are formed through detection of acoustic waves generated by rapid thermoelastic expansion caused by optical absorption of short laser pulses<sup>21</sup>. A focused ultrasonic transducer detects one-dimensional depth-resolved signals (or A-lines), and cross-sectional images (or B-scans) are produced by rotating a scanning mirror that directs both optical and acoustic waves. The endoscope system records and displays a set of dual wavelength photoacoustic and ultrasonic B-scan images in real time during the constant rotation (~4 Hz) of the mirror. By interleaving two optical pulses of different wavelengths and one acoustic pulse at each angular step of the mirror, spatially coincident images are recorded from the generated photoacoustic and ultrasonic A-line signals, even during periods of substantial motion of the target. Volumetric data sets are acquired by recording sequential A-line data during the constant rotational motion of the mirror and mechanical pullback of the probe at a speed of ~200  $\mu\text{m s}^{-1}$ . Oxy- and deoxyhemoglobins, two of the dominant absorbers of visible light in most soft biological tissue, have different absorption spectra. With dual-wavelength photoacoustic imaging, it is possible to calculate total hemoglobin concentration and  $\text{sO}_2$  values (**Supplementary Fig. 3**)<sup>20,24</sup>.

## Upper gastrointestinal tract imaging *in vivo*

We imaged the esophagi of four adult New Zealand white rabbits (**Supplementary Fig. 4**) and collected 5–10 volumetric data sets from each rabbit without complications. We recorded about 2,800 B-scan slices at ~50- $\mu\text{m}$  intervals for each volumetric set of PAT at 562 nm, PAT at 584 nm and ultrasonic imaging. The endoscopic system provides anatomic information of the rabbit's esophagus, surrounding tissue and proximal organs, covering an approximately 14-cm-long,

18-mm-diameter volume (Fig. 2 and **Supplementary Video 2**) within a scanning time of ~12 min. Volume renderings enable three-dimensional (3D) visualization of the morphology and configuration of organs surrounding the esophagus with both contrast mechanisms (Fig. 2a–c and **Supplementary Video 3**). We created the photoacoustic image (Fig. 2a) from data acquired at a 584-nm laser wavelength in which the photoacoustic signal is proportional to the total hemoglobin concentration. Both the photoacoustic (Fig. 2a) and ultrasonic (Fig. 2b) images show the lung and trachea profiles where they abut the esophagus; however, only the photoacoustic image (Fig. 2a) provides peripheral vasculature information of these organs. Through transesophageal imaging, the tomographic endoscope obtained cross-sectional images of the lung with optical absorption-based contrast (Fig. 2d) and acoustic reflectance-based contrast (Fig. 2e). We found obvious differences in the locations of the contrast origins for the two imaging modes (Fig. 2f). When the endoscope passed by the trachea, we observed strong photoacoustic (Fig. 2g) and ultrasonic (Fig. 2h) signals around the contact point between the esophagus and trachea, which run parallel (Fig. 2i) from the cricoid cartilage to the carina. The probe's limited angular FOV covers only ~270°. To obtain a full 360° view, we reinserted the probe with 180° rotation and imaged the dorsal region of the esophagus. These results are shown in **Supplementary Figure 5** and **Supplementary Video 4**.

For a straightforward comparison of the volumetric images over the entire 360° view, we combined the ventral (Fig. 2) and dorsal (**Supplementary Fig. 5**) volumetric data sets, using the method described in **Supplementary Figure 6**, and produced three radial-maximum amplitude projection (RMAP) images, which show the total hemoglobin distribution (Fig. 3a), the  $\text{sO}_2$  level (Fig. 3b) and the ultrasonic echogenicity distribution (Fig. 3c), respectively. To produce the  $\text{sO}_2$  distribution over the scanned area (Fig. 3b), we spectrally analyzed dual-wavelength photoacoustic data sets (**Supplementary Fig. 3**). The 584-nm photoacoustic image (Fig. 3a) shows the total hemoglobin distribution in the lung, trachea and surrounding blood vessels. Also, the aorta and caudal vena cava are shown in the image (Fig. 3a) and are distinguished from each other by differences in  $\text{sO}_2$  (Fig. 3b). As confirmed by dissection after imaging (**Supplementary Fig. 7**), the heart was located near the mid-ventral line between the two lobes of the lung, and several prominent blood

**Figure 1** Illustration of simultaneous, multiwavelength photoacoustic (PA) and ultrasonic (US) endoscopy. (a) The endoscope carries out circumferential sector scanning by rotating a scanning mirror, which reflects both the ultrasonic waves and laser pulses and enables static mounting of the associated illumination and ultrasonic pulse-generation detection units. At each angular step of the mirror (~1.42°), both the first ( $\lambda_1$ ) and second ( $\lambda_2$ ) pulsed laser beams are independently fired through the optical fiber and the acoustic pulse is generated by the ultrasonic transducer with a constant time delay of ~30  $\mu\text{s}$  between each of the laser and acoustic pulses. The ensuing photoacoustic and ultrasonic echo waves are detected and converted into electric signals by the ultrasonic transducer; the signals are then recorded and displayed on a computer. The 30- $\mu\text{s}$  time delay is necessary to ensure that the sample has relaxed and that consecutive signals will not interfere with each other. However, this delay does not substantially affect co-registration of the three images because the difference between the angular positions of each signal is small and is well within the spatial resolution of the detector. (b) A photo shows the side-scanning 3.8-mm-diameter probe prototype firing a 562-nm laser beam. Scale bar, 2 cm. (c) Definition of Cartesian and cylindrical coordinate systems. The  $z$ -axis is defined along the endoscope axis (or pullback direction). (d) A volumetric image comprising consecutive B-scan slices. (e) A representative cross-section of d along the  $x$ - $y$  plane, which shows the endoscope's 270° angular FOV.

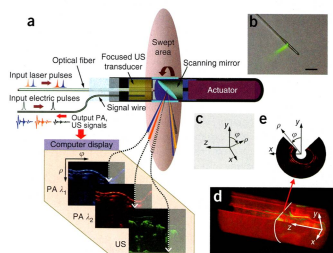
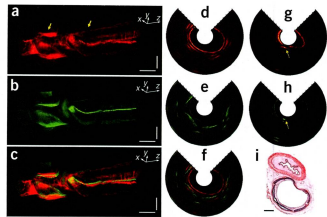


**Figure 2** Simultaneous, co-registered, PAE-EUS pseudocolor images from a rabbit esophagus *in vivo*. (a) Three-dimensionally rendered photoacoustic structural image. The left- and right-hand sides of this image correspond to the lower and upper esophagus, respectively, and the lower portion ( $-y$  axis) to the ventral side of the rabbit. To more clearly show the structures surrounding the esophagus, we excluded signals generated from the esophagus. (b) Co-registered ultrasonic structural image for the same volume of a. (c) An overlaid image of a and b. In a–c, the horizontal and vertical scale bars represent 2 cm and 5 mm, respectively. (d) A representative photoacoustic  $x$ - $y$  cross-sectional image (18 mm in diameter) near the lung, as indicated by the left arrow in a. The  $\sim 0.8$ -mm-thick region from the inner surface of the imaged lumen represents the signals from the esophagus. The surface of the lung is shown  $\sim 1$  mm from the inner surface. (e) Corresponding ultrasonic cross-sectional image of d. (f) A combined image of d and e. (g) A representative photoacoustic  $x$ - $y$  cross-sectional image (18 mm in diameter) near the trachea, as indicated by the right arrow in a. (h) Corresponding ultrasonic cross-sectional image of g. In g and h, the dotted arrows indicate the contact point between the trachea and the esophagus. In d–h, the hash marks represent 1-mm intervals. (i) Representative histology (H&E stain) of the esophagus (top) and trachea (bottom). Scale bar, 1 mm.



vessels (see the arrow in Fig. 3a), including the aorta and caudal vena cava, branched out from the heart. The aorta and caudal vena cava show reasonable  $sO_2$  values of  $\sim 95\%$  and  $\sim 70\%$ , respectively (Fig. 3b). The adventitial surface of the trachea and the network of the prominent blood vessels distributed in the apical lung pleura (Fig. 3b) show relatively high  $sO_2$  because they directly receive fully oxygenated blood from the heart. Strong ultrasonic contrast was generated at the lung's surface and the trachea (Fig. 3c), which contains cartilage (Fig. 2i). The image reproducibility of the endoscope is presented in Supplementary Figure 8.

#### Lower gastrointestinal tract and lymphatic system imaging *in vivo*

We imaged the lower gastrointestinal tracts of Sprague-Dawley rats using the same scanning parameters (Supplementary Fig. 9). Within a scanning time of  $\sim 4.6$  min, we imaged the descending colon of a rat over a section  $\sim 5.5$  cm in length and  $\sim 12$  mm in diameter. Volumetric images clearly show the lumen's architecture (Fig. 4a and Supplementary Video 5). Photoacoustic imaging shows densely distributed blood vasculature in the colon wall, whereas ultrasonic

imaging shows the density of tissue in the mucosal and submucosal layers and other mesenteric tissues entangled around the tract (Fig. 4a). Compared with single-mode EUS<sup>1,6</sup>, PAE-EUS provides high-resolution vasculature information in the gastrointestinal tract and clearly shows optical and mechanical contrast differences. The corresponding  $sO_2$  map (Fig. 4b) shows the oxygen saturation variation in the imaged blood vessels. The ultrasonic RMAP image (Fig. 4c) shows hyperechoic regions generated by the lumen wall and exterior tissues. The photoacoustic images also show strong signals from blood vessels; however, corresponding ultrasonic signals are very weak at those locations (Fig. 4d,e). Although the ultrasonic imaging provided an approximate boundary profile of the colon, its overall contrast is weak owing to the low echogenicity of the soft mucosal tissues (Fig. 4f).

To demonstrate lymphangiographic imaging, we imaged parts of the lymphatic system near the lower gastrointestinal tracts in Sprague-Dawley rats (Supplementary Fig. 10). Accumulation of Evans blue dye provided the photoacoustic image contrast for the lymph nodes and vessels, which are not intrinsically optically absorptive in the visible spectrum. We targeted lymph nodes near the dorsal zone of the descending colon. In these experiments, we used 523-nm light to image the surrounding blood vasculature and 640-nm light to image the accumulation of Evans blue in the lymphatic system, which has an absorption peak at  $\sim 610$  nm (Supplementary Fig. 3). After recording a set of control images, we injected  $\sim 0.4$  ml of Evans blue dye (0.3%) in the inner legs of the rats, and then acquired serial volumetric

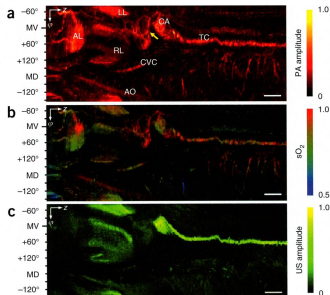
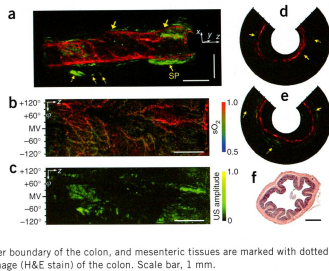


**Figure 3** RMAP images over a full  $360^\circ$  angular FOV (views from the inside of the esophagus). The left- and right-hand sides of these three images correspond to the lower and upper esophagus, respectively. (a) Normalized photoacoustic RMAP image showing the total hemoglobin distribution, with the esophageal signals excluded during the RMAP construction. AL, accessory lobe; LL, left lobe; RL, right lobe of the lung; AO, aorta; CVC, caudal vena cava; CA, carina; TC, trachea. (b) Functional photoacoustic RMAP image showing the  $sO_2$  levels of the imaged structures in a. (c) Normalized ultrasonic RMAP image showing the echogenicity distribution. In each image, the vertical  $\phi$  axis corresponds to the angular range of  $360^\circ$ , and the horizontal  $z$  axis corresponds to the pullback length of 14 cm. The approximate mid-ventral (MV) position and angular measures from the MV position are marked along the vertical  $\phi$  axis; the positive and negative values correspond to the right and left sides of the animal and MD denotes the mid-dorsal position. Scale bars, 1 cm (for the horizontal direction only).

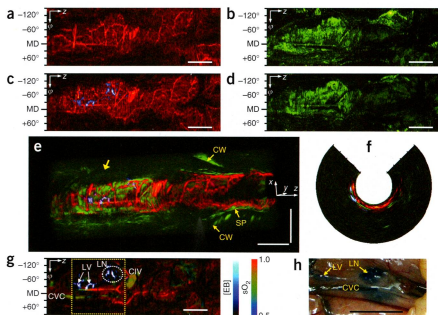
**Figure 4** Simultaneous, co-registered, PAE-EUS colonoscopic pseudocolor images of a rat colon acquired *in vivo*. **(a)** Three-dimensionally rendered photoacoustic-ultrasonic structural image. The right side of this image is closer to the anus, and the negative  $y$  axis corresponds to the ventral direction of the animal. The red and green colors correspond to photoacoustic and ultrasonic signals, respectively. The dotted arrows indicate mesenteric tissue entangled around the tract, and SP denotes the sphincter. Scale bars, 1 cm (horizontal) and 5 mm (vertical). **(b)** Photoacoustic RMAP image showing the  $sO_2$  levels of the imaged structures in A (views from the inside of the colon). **(c)** Corresponding to **(b)** ultrasonic RMAP image showing the echogenicity distribution. In **b** and **c**, the vertical  $\phi$  axis corresponds to the angular FOV covering  $270^\circ$ , and the horizontal  $z$  axis corresponds to the pullback length of 5.5 cm. The approximate mid-ventral (MV) position and angular measures from the MV position are marked along the vertical  $\phi$  axis, where the positive and negative values correspond to the right and left sides of the rat. Scale bars, 1 cm (for the horizontal direction only). **(d,e)** Photoacoustic-ultrasonic cross-sectional images from the position indicated by the left and right arrows in **a**, respectively. In **d** and **e**, the solid arrows indicate the outer boundary of the colon, and mesenteric tissues are marked with dotted arrows. The hash marks represent 1-mm intervals. **(f)** A typical histology image (H&E stain) of the colon. Scale bar, 1 mm.



data sets every 20 min for ~1 h (Supplementary Fig. 11). In each instance, we imaged a volume of ~7.2 cm in length and 12 mm in diameter, with a scanning time of ~8 min. Serial photoacoustic RMAP images acquired at 640 nm showed an obvious photoacoustic signal increase and dynamic changes of the Evans blue distribution in the lymphatic system, and spectroscopic analysis at the two wavelengths enabled accurate differentiation between the blood and lymph signals (Supplementary Fig. 11). We present a set of photoacoustic and ultrasonic images containing both pre- (Fig. 5a,b) and postcontrast injection images (Fig. 5c,d), which illustrates the capability of contrast-enhanced photoacoustic imaging of the local lymphatic system. We observed the strongest Evans blue signals in the photoacoustic RMAP image (Fig. 5c) acquired ~40 min after Evans blue injection; they are clearly different from those in the control photoacoustic RMAP image

(Fig. 5a). However, the corresponding ultrasonic image (Fig. 5d) did not show any notable changes from the control image (Fig. 5b). Volume rendering of the co-registered dual-wavelength photoacoustic and ultrasonic signals enables 3D visualization of the lymphoacoustic structure (PAE) inside a density map (EUS) of the tissue (Fig. 5e, Supplementary Videos 6 and 7). As shown in a cross-sectional image (Fig. 5f), the lymph nodes are in contact with the outer colon wall. A spectrally processed photoacoustic RMAP image (Fig. 5g), which excludes signal from the colon, clearly shows the  $sO_2$  distribution and lymph structures in the imaged anatomy outside the colon and provides an effective comparison with the surgical photo (Fig. 5h and Supplementary Fig. 12). Notably, PAE detected signals from lymph vessels as small as ~1 mm in diameter, far exceeding the current capabilities of EUS<sup>1,5,7,8</sup>.

**Figure 5** Lymphovascular system imaging *in vivo* near a rat colon. **(a)** Control photoacoustic RMAP image before Evans blue (EB) administration (views from the inside of the colon). The right side of this image is closer to the anus. **(b)** Co-registered ultrasonic RMAP image of **a**. **(c)** Photoacoustic RMAP image at ~40 min after injection (processed from the dual-wavelength data). The red and blue colors represent photoacoustic signals from blood and Evans blue, respectively. **(d)** Corresponding ultrasonic RMAP image of **c**. In **a–d**, the vertical  $\phi$  axis corresponds to the angular FOV covering  $270^\circ$ , and the horizontal  $z$  axis corresponds to the pullback length of 7.2 cm. The approximate mid-dorsal (MD) position and angular measures from the MD position are marked along the vertical  $\phi$  axis. **(e)** Merged volumetric image of **c** and **d**. SP, sphincter; CW, cavity wall. **(f)** A representative  $x$ - $y$  cut near the location indicated by the arrow in **e**. The blue-colored regions represent a lymph node. **(g)** A spectrally processed photoacoustic RMAP image from the data set shown in **c**, with the colon signals excluded. This image shows the lymph structures and  $sO_2$  distribution of the surround vasculature outside the colon. The arrows indicate lymph vessels (LV), and the dotted circle corresponds to the left lumbar node (LN) in **h**. CVC, caudal vena cava; CIV, left common iliac vein. **(h)** A postimaging surgical photo showing the stained lymph node and vessels located outside the colon. This area approximately corresponds to the yellow dotted square in **g**. Scale bars, 1 cm (horizontal) and 5 mm (vertical).





## DISCUSSION

PAE's unique combination of optical absorption contrast, wide FOV and great penetration depth suggests many possibilities for medical applications involving diagnostic or interventional procedures. Most notably, PAE can be realized simply through a few modifications and the addition of a light-delivery system to EUS technology while conserving EUS capabilities. The simultaneous, spatially coincident photoacoustic and ultrasonic dual-mode imaging provides unprecedented information and promotes morphologic and functional understanding of the target tissue. In the upper gastrointestinal tract images (Fig. 2), we observed organs surrounding the esophagus, such as the lung and trachea, in both the photoacoustic and ultrasonic images; however, only photoacoustic images showed their adjacent vasculatures. Although contrast-enhanced ultrasonic techniques<sup>1,9,10</sup> such as Doppler ultrasound<sup>1,31</sup> are capable of imaging blood vessels, these techniques' sensitivities are all much lower than that of PAT, which has recently achieved sufficient signal sensitivity to detect single red blood cells<sup>32</sup>. Additionally, photoacoustic imaging provides functional information with endogenous contrast (Figs. 2–4), and, with the aid of an exogenous contrast agent, it can also provide information about the dynamics of regional lymph systems (Fig. 5 and Supplementary Fig. 11). These experimental results show the dual-mode endoscope's transenteric deep imaging ability and its complementary contrast production.

PAE signal detection is predicated on the same basic principles as EUS signal detection, and the required components for light delivery do not substantially affect the probe's endoscopic functionality. Therefore, this integrated endoscopic technique could be translated into virtually all current application areas of EUS<sup>1,2</sup>. Added angiographic and spectral imaging functions would enhance EUS's role in addressing the aforementioned high-mortality cancers and in many other clinical applications. From diagnosis to therapy, the possible contributions of PAE are conceived as follows. First, PAE's strong spectroscopic imaging ability and high optical contrast could promote incidental findings of disease symptoms in many routine endoscopic procedures. Tumor detection with conventional single-mode EUS has been a challenge because of the similar mechanical properties between diseased and healthy soft tissue, as well as between blood and lymph vessels. PAE's spectral imaging ability, based on physiologically-specific endogenous optical contrasts, yields vital functional information, which might enable earlier detection of tissue abnormalities. Additionally, on the basis of the lymphovascular information that PAE can provide, it might improve the accuracy of cancer staging. Contrast agents for EUS are limited, whereas PAE's unique broad-spectrum, absorption-based contrast fosters the use of various kinds of contrast agents<sup>24–26</sup>, thus enabling many applications, such as lymph node mapping<sup>23,26</sup> and molecular imaging for cancer mapping and staging<sup>12,24,25,33</sup>. Finally, PAE's high contrast and high resolution vasculature imaging ability could enable more advanced interventional procedures by helping practitioners guide devices to extract samples for biopsy or to inject drugs for chemotherapy.

Another key feature of PAT is its exceptional scalability<sup>21</sup>, spanning from providing subcellular resolution at shallow depths to providing optical contrast of tissue several centimeters deep with ultrasound-level spatial resolution. For lumen wall imaging in which great penetration depth is not necessary, optically resolved photoacoustic imaging could provide greater spatial resolution<sup>32</sup>. In many clinical transenteric imaging applications, deep imaging ability is preferable to shallow high-spatial-resolution imaging. By choosing a lower-center-frequency transducer, red-shifting laser wavelengths to the

near-infrared spectrum or increasing the laser pulse energy by enlarging the illumination area, the penetration depth of PAE can be greatly improved, thus making it more applicable to current clinical requirements (see **Supplementary Discussion**). In this study, we used a pair of laser beams at 584 nm and 562 nm to determine the distributions of total hemoglobin concentration and  $\text{sO}_2$ , and we used another pair at 640 nm and 523 nm to image the lymphovascular system with the aid of a contrast agent. However, one can freely choose illumination wavelengths, from ultraviolet to near-infrared, to quantitatively evaluate other cellular or physiological constituents, such as cell nuclei<sup>34</sup> or hydration levels<sup>35</sup>. Further, more laser pulses can be added to each angular step of the mirror to generate richer spectral information for a thorough characterization of diseased tissues. With the integration of PAE, EUS could become a powerful clinical tool.

## METHODS

Methods and any associated references are available in the online version of the paper.

*Note: Supplementary information is available in the online version of the paper.*

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## AUTHOR CONTRIBUTIONS

J.-M.Y. built the system, did the experiments, and wrote the manuscript. C.F. developed the data acquisition program, carried out the experiments, and co-wrote the manuscript. R.C., Q.Z., and K.K.S. designed and fabricated the ultrasonic transducers. J.Y. contributed to the data processing algorithms and also assisted with data processing and experiments. X.C. helped with the experiments. K.M. contributed to the system development. L.V.W. directed the project, conceived the endoscope design, discussed the experiments and revised the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available at <http://www.nature.com/doi/funder/10.1038/nm.2823>.

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**Erratum:** Fast-degrading elastomer enables rapid remodeling of a cell-free synthetic graft into a neoartery

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The above Technical Report was inadvertently omitted from the print version of the July 2012 issue of the journal. The final version of the article follows this note.

# Fast-degrading elastomer enables rapid remodeling of a cell-free synthetic graft into a neoartery

Wei Wu<sup>1,2</sup>, Robert A Allen<sup>1</sup> & Yadong Wang<sup>1,3,4</sup>

Host remodeling is important for the success of medical implants, including vascular substitutes. Synthetic and tissue-engineered grafts have yet to show clinical effectiveness in arteries smaller than 5 mm in diameter. We designed cell-free biodegradable elastomeric grafts that degrade rapidly to yield neoarteries nearly free of foreign materials 3 months after interposition grafting in rat abdominal aorta. This design focuses on enabling rapid host remodeling. Three months after implantation, the neoarteries resembled native arteries in the following aspects: regular, strong and synchronous pulsation; a confluent endothelium and contractile smooth muscle layers; expression of elastin, collagen and glycosaminoglycan; and tough and compliant mechanical properties. Therefore, future studies employing large animal models more representative of human vascular regeneration are warranted before clinical translation. This cell-free approach represents a philosophical shift from the prevailing focus on cells in vascular tissue engineering and may have an impact on regenerative medicine in general.

A key challenge of arterial substitutes is that they need to withstand arterial pressure immediately upon implantation<sup>1</sup>. Thus, the classic approaches to arterial substitutes use strong materials. This is reflected in autografts, synthetic grafts and many tissue-engineered grafts<sup>1–7</sup>. Tissue-engineered grafts typically show limited host cell infiltration and remodeling even 6–12 months after implantation<sup>8–11</sup>. In vascular extracellular matrix (ECM), collagen provides strength and elastin provides elastic recoil. Collagen expression is typically very high for existing vascular grafts, whereas elastin expression is usually low<sup>12,13</sup>. Notably, host remodeling of tissue-engineered constructs after implantation can increase elastin expression<sup>14</sup>. This positive remodeling suggests that the host may be a good source of cells and a more efficient ‘bioreactor’ than the current *in vitro* tissue-engineering paradigm. This inspired us to fully harness the body’s regenerative capacity to remodel cell-free synthetic grafts with an open porous structure that accelerates cell infiltration and remodeling (Fig. 1a). We believe that, for a graft that can withstand arterial pressure, host remodeling capability is the most important criterion.

Rapid remodeling of a synthetic graft to a neoartery would most likely offer efficient integration with host tissue, a nonthrombogenic

lumen and mechanical properties matching native vessels because it reduces the duration of host exposure to foreign materials. Enabling application of synthetic vascular grafts in small-diameter arteries offers multiple advantages: avoidance of donor-site morbidity, bypass of *in vitro* cell culture, ready availability, easy storage and transport, and potentially faster clinical adoption.

To design a synthetic graft capable of rapid host remodeling, we examined the following criteria: First, for the graft material we chose a fast degrading elastomer, poly(glycerol sebacate) (PGS), because timely degradation is essential for rapid host remodeling and mechanical conditioning is recognized as an important remodeling cue<sup>6</sup>. Elastomers efficiently transduce mechanical stimulation to cells. Second, for graft porosity, we chose highly porous grafts with interconnected pores to enable immediate host cell infiltration. To prevent blood cell loss, we enclosed grafts with a dense, nonwoven sheath. Last, for thromboresistance, we chose heparin coating. The graft is a blood-contacting foreign material prone to clotting, which can occlude grafts and block cell infiltration into the graft wall. Heparin is a well-recognized standard in thromboresistance<sup>15</sup>.

## RESULTS

### The open porous graft is suturable and resists platelet adhesion

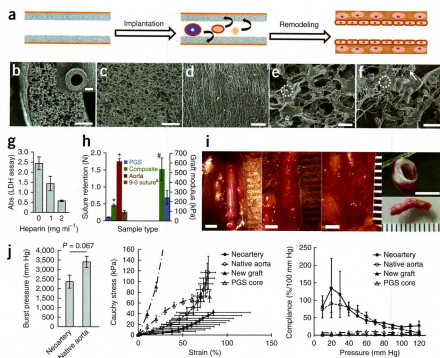
Consistent with the design criteria, our graft consists of a heparin-coated porous tube wrapped with a 15- $\mu$ m-thin electrospun sheath (Fig. 1b–d). The porous tube is made from PGS fabricated as previously described except a 1-mm mandrel was used<sup>13</sup>. The sheath is a polycaprolactone (PCL) mesh that increases graft strength and prevents bleeding by controlled fibrin formation within the sheath. Composite grafts are stored at ambient temperature. Microcomputed tomography (microCT) morphometric analysis indicates that the inner diameter of the graft is 720  $\mu$ m, the wall thickness is 290  $\mu$ m and over 99.99% of the pores are interconnected (Supplementary Table 1). High pore interconnectivity is crucial for efficient cell infiltration, the first step in initiating host remodeling. PGS has numerous hydroxyl groups that can form hydrogen bonds with heparin. Thus, heparinization of PGS most likely renders the graft lumen nonthrombogenic and inhibits excessive blood clotting within the graft wall. Heparin coating significantly ( $P < 0.0001$ ) reduces platelet adhesion on grafts (Fig. 1e–g). Scanning electron microscopy (SEM) reveals that

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**Figure 1** Characterization of the composite graft and host remodeling of the graft *in vivo*.

(a) Schematic representation of direct implantation of the cell-free graft and the proposed remodeling process of the graft into a biological neoartery. (b) SEM images of composite grafts to show surface topology; scale bar, 100  $\mu\text{m}$ . Inset: top view of the graft; scale bar, 500  $\mu\text{m}$ . (c) Lumen of the PGS tube; scale bar, 100  $\mu\text{m}$ . (d) The PCL fibrous sheath; scale bar, 20  $\mu\text{m}$ . (e) SEM of heparin-soaked PGS after incubation in platelet-rich plasma to show platelet adhesion and fibrin deposition. Scale bar, 10  $\mu\text{m}$ . (f) SEM of unheparinized PGS after incubation in platelet-rich plasma; scale bar, 10  $\mu\text{m}$ . An adherent platelet is circled in e and f to highlight difference in platelet morphology. Arrow indicates fibrin in f. (g) Platelet adhesion on PGS presoaked in varying heparin concentrations, quantified by lactate dehydrogenase assay ( $n = 5$ ).  $P < 0.0001$  between all groups. Abs, absorbance. (h) Suture retention and elastic moduli of grafts.  $\Delta$ , break force of 9-0 suture.  $P < 0.05$ ; \*, composite graft versus 9-0 suture; +, composite graft versus rat aorta; #, composite graft modulus ( $n = 8$ ) versus that of PGS (grafts without the PCL sheath,  $n = 3$ ).  $n = 5$  for other groups. (i) Gross appearance of the graft during host remodeling to assess integration with host tissue. From left to right: day 0, day 14, day 90 and day 90 (top and bottom). Scale bars, 2 mm; all ruler ticks 1 mm. Nondegradable sutures (black) mark the graft location. (j) Mechanical properties of neoarteries. Left, the burst pressure of the neoartery ( $n = 3$ ) and native aorta ( $n = 4$ ). Middle, stress-strain curves of arteries and new grafts. 'New grafts' represents unimplanted composite grafts. 'PGS core' represents new grafts without the PCL sheath. Right, compliance of arteries and new grafts. Standard errors for new grafts and the PGS core are very small and barely visible at the plotted scale. For j,  $n = 3$  for neoarteries and  $n = 4$  for native aortas, new grafts and PGS cores. Data represent mean  $\pm$  s.d. for g,h and mean  $\pm$  s.e.m. for j.



heparin coating (2 mg ml<sup>-1</sup>) also reduces fibrin formation, and most adhered platelets appear quiescent (Fig. 1e,f). The small pore size of the PCL sheath probably prevents fibrin formation within the sheath and prevents leakage. Although very thin, the sheath significantly increases the suture retention force of the graft from 0.11  $\pm$  0.0087 to 0.45  $\pm$  0.031 N and is stronger than the break force of the 9-0 suture (0.26  $\pm$  0.046 N) used for the microsurgical anastomosis (Fig. 1h). Furthermore, the PCL sheath increases elastic tensile modulus from 243  $\pm$  71.8 kPa to 536  $\pm$  119 kPa ( $P < 0.05$ ) and ultimate tensile strength from 76.6  $\pm$  15.7 kPa to 3,790  $\pm$  1,450 kPa ( $P < 0.001$ ).

### Rapid graft remodeling leads to strong and compliant neoartery

Interposition grafting of ethylene oxide-sterilized grafts was performed in the abdominal aorta of Lewis rats ( $n = 21$ ) without heparin administration during surgery or systemic heparin treatment after (Fig. 1i). The implantation immediately exposed the grafts directly to 120 mm Hg pressure and an arterial hemodynamic environment. Therefore, in terms of allowing unobstructed blood flow upon implantation, the grafts performed the same function as autografts in bypass surgeries. Grafts reddened with the infiltration of blood cells, but the PCL sheath effectively prevented bleeding. The host rapidly remodeled patent grafts by 14 d after grafting, grafts were more translucent and compliant and began to integrate with the surrounding host tissue; by 90 d, grafts were covered with fascia, closely resembled the native aorta and were well integrated with host tissue (Supplementary Video 1).

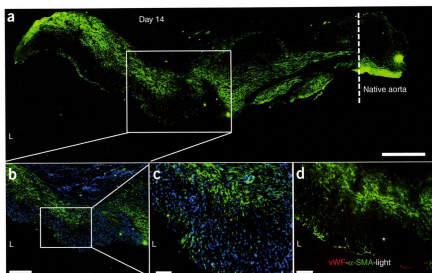
Accompanying the visual remodeling is the pronounced change in mechanical properties. Neoartery burst pressure is 2,360  $\pm$  673 mm Hg, approaching that of native aorta at 3,415  $\pm$  529 mm Hg (Fig. 1j)

and considerably higher than that of human saphenous veins (1,680  $\pm$  307 mm Hg), the most used autografts for coronary artery bypass surgery and lower-limb vascular reconstruction<sup>16,17</sup>. Unimplanted grafts leak due to their high porosity and have no burst pressure to compare. Before implantation, composite grafts were stiffer than native arteries, with no toe region in the stress-strain curve (Fig. 1j). Host remodeling substantially altered the stress-strain curve of grafts with a clear toe region in the neoarteries. Neoarteries appeared softer than the native aorta, although the differences were insignificant. More notably, the compliance of the neoarteries (11%  $\pm$  2.2%) is statistically the same as native aorta at 6.7%  $\pm$  2.3% in the 80–120 mm Hg range<sup>4</sup>. More informative than this single value of compliance is a plot of compliance over the whole pressure range (Fig. 1j). Of note, the neoartery was not only strong but also compliant. The high compliance of neoarteries contrasts with the lower compliance of unimplanted grafts, suggesting extensive graft remodeling.

This fast host remodeling is explained by widespread cell infiltration soon after implantation. The open porous structure of the graft allows extensive cell penetration into the graft wall, and nucleated cells occupied many of the pores within 3 d (Supplementary Fig. 1). Smooth muscle cells extensively infiltrated the graft within 14 d (Fig. 2a). Cells positive for  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), a protein specific to mural cells including smooth muscle, were widely distributed within the graft wall. Higher magnification illustrated that smooth muscle cells were not organized into circumferential layers at this early stage and were mixed with  $\alpha$ -SMA-negative cells (Fig. 2b,c). Co-staining of endothelial and smooth muscle cells indicated that the smooth muscle layer was separated from the blood by an endothelium



**Figure 2** Smooth muscle cell infiltration and organization at 14 d. (a) Smooth muscle cell distribution ( $\alpha$ -SMA, green) within the remodeled graft wall. The tissue was split longitudinally, half of which is shown. Native aorta is on the right; its border with the graft is indicated by the dashed line; scale bar, 500  $\mu$ m. L, lumen. (b) Magnified view of the mid-graft shows distribution of both  $\alpha$ -SMA-positive (green) and  $\alpha$ -SMA-negative cells. Nuclei counterstained by DAPI (blue); scale bar, 250  $\mu$ m. (c) Further magnification of the mid-graft to view the complicated smooth muscle cell distribution ( $\alpha$ -SMA, green); scale bar, 50  $\mu$ m. (d) Distribution of endothelial cells (VWF, red) and smooth muscle cells ( $\alpha$ -SMA, green) in the graft wall. Immunofluorescent images merged with the bright-field image (darkened to not overwhelm the fluorescent images). Dark spots (\*) in the bright-field image might be residual graft material. Scale bar, 100  $\mu$ m. Bright-field images of original brightness are in **Supplementary Figure 2**.

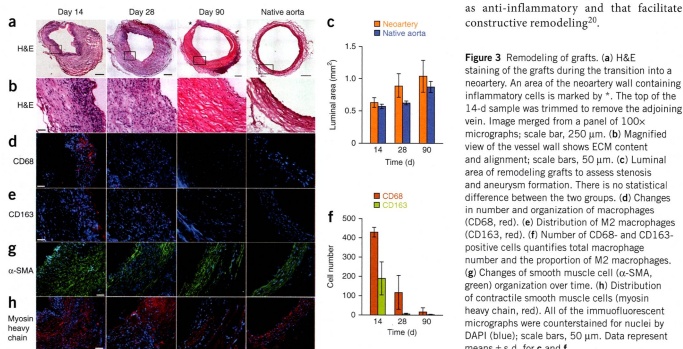


(Fig. 2d). By 90 d, the cellularity of the neoartery was slightly higher than but comparable to that of native aortas ( $2.14 \pm 0.34 \mu$ g DNA per mg wet weight versus  $1.36 \pm 0.40 \mu$ g DNA per mg wet weight,  $P = 0.06$ ). Bright-field images revealed a band of dark spots mixed with cells located generally closer to the lumen. This acellular matter is probably remnants of graft materials that are visible as dark fibers in bright-field images (**Supplementary Fig. 2**).

Profound graft remodeling is also reflected at the tissue level in histological observations. Within 14 d, grafts experienced extensive degradation (Fig. 3a,b), whereas cells synthesized substantial ECM (Fig. 3b). Therefore, remodeled grafts withstand arterial pressure despite substantial graft degradation. The luminal area of the graft and the native aorta remained statistically the same throughout remodeling, suggesting absence of aneurysm and stenosis (Fig. 3c). The thickness of the graft wall decreased over time but was still thicker than the native

aorta at 90 d. The majority of the graft resembled native arteries, albeit the ECM fibers were less dense and less organized (Fig. 3b). Both the difference in wall thickness and ECM fiber density demonstrate that progressive remodeling of the graft is still active at 3 months.

Macrophages actively participate in the remodeling of the graft: a band of inflammatory cells, including newly recruited macrophages (CD68<sup>+</sup>), was visible beneath the lumen at 14 d. At 28 d, macrophages were distributed more evenly throughout grafts at a lower density than at 14 d (Fig. 3d–f). At 90 d, most of the inflammatory response was resolved, with only a small area of the neoartery positive for macrophages (Fig. 3d–f). This trend correlates with the disappearance of the putative graft materials (**Supplementary Fig. 2**). Macrophages are heterogeneous, comprising proinflammatory (M1) and several alternatively activated (M2) subclasses<sup>18,19</sup>. Positive staining for CD163 (Fig. 3e) suggests the presence of M2 macrophages that are generally accepted as anti-inflammatory and that facilitate constructive remodeling<sup>20</sup>.



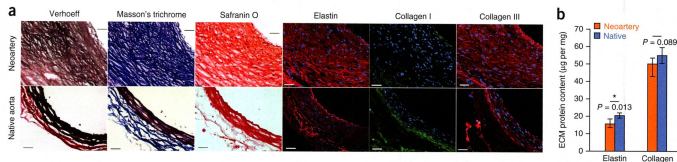


**Figure 4** ECM organization and quantification at 90 d. (a) Verhoeff's, Masson's trichrome and safranin O staining show elastin (black), collagen (blue) and glycosaminoglycans (red), respectively. Immunofluorescent staining shows distribution of elastin (red), collagen I (green), and collagen III (red). Top: day 90 explant; bottom: native aorta. Scale bars, 50  $\mu$ m. (b) Quantification of elastin ( $n = 4$ ) and total collagen ( $n = 4$ ). Data represent mean  $\pm$  s.d.

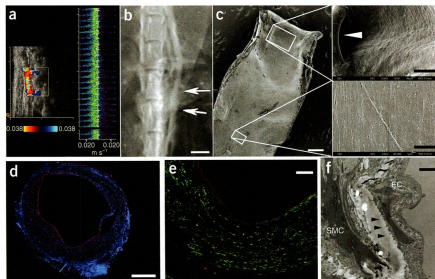
Smooth muscle cells are important for blood vessel function. Immunofluorescent staining revealed the presence of smooth muscle cells within 14 d and a progressively more organized media layer (Fig. 3g,h). Myosin heavy chain is a late-stage differentiation marker for smooth muscle. Strong expression of myosin heavy chain indicated a contractile smooth muscle phenotype in neoaortas (Fig. 3h). Neoaortas stained positive for fibroblast surface protein in the outer layer at 90 d, suggesting the formation of an adventitia-like tissue (Supplementary Fig. 3). The layers were distinct, resembling the trilaminar structure of a muscular artery.

The marked impact of host cells on the graft is further revealed in ECM composition. The neoaortay wall contained substantial amounts of elastin, collagens I and III and glycosaminoglycans (Fig. 4a). The amount of elastin in the neoaortay at 90 d was 77% of that in native aorta, and the amount of total collagen was statistically the same as native aorta (Fig. 4b). These ECM macromolecules aligned circumferentially, mimicking their orientation in native arteries. However, native matrix is more compact and less cellular than in the neoaortay. Nonetheless, high ECM production provides a molecular explanation of the observed match of mechanical properties between the neoaortay and the host aorta.

**Endothelialized neoaortay pulses synchronously with host aorta**  
Integration with host tissue is often a major challenge in tissue engineering. Laser Doppler ultrasound imaging of the grafts indicated uninhibited blood flow and strong, synchronous pulsation with host

aorta (Fig. 5a and Supplementary Video 1). Regular and clear pulsation of neoaortas indicates thorough integration with host tissue. To the best of our knowledge, this is the first report of any vascular graft that pulses with host arteries. The high patency was corroborated by angiography (Fig. 5b), correlating well with a confluent endothelium that transitioned smoothly from neoaortay to host aorta. The transition was marked only by the suture (Fig. 5c). von Willebrand factor (vWF) staining revealed a confluent endothelial monolayer covering the lumen (Fig. 5d,e). A basement membrane separated the endothelium from the smooth muscle layer, as indicated by transmission electron microscopy (Fig. 5f). Basement membrane prevents smooth muscle cell migration to the endothelial layer, thereby preventing intimal hyperplasia<sup>21</sup>. We found no evidence of intimal hyperplasia in neoaortas. In the four observed cases of graft occlusion (4/21; 19.0%) the cause was acute anastomotic thrombosis in three cases where the rats died. Acute thrombosis was also suspected in one rat that survived with an occluded graft, because no graft remodeling was observed in the explant (Supplementary Fig. 4). Acute thrombosis is likely to be due to endothelial injury at the anastomosis in the absence of systemic anticoagulation<sup>22</sup>. Overall patency as determined by ultrasonography, angiography and necropsy was 80.9% (17 patent grafts/21 total grafts) at time points of up to 90 d. Patency rates were 60% (3/5), 100% (5/5) and 81.8% (9/11) for grafts explanted at days 14, 28 and 90, respectively. This overall graft patency is similar to a previous report of an antithrombogenic vascular graft<sup>7</sup>.

**Figure 5** Endothelialization of the neoaortay and vascular patency at day 90. (a) Laser Doppler ultrasound imaging assessment of graft patency and synchronization of pulsation with adjacent host aorta. Synchronization of pulsation also shown in Supplementary Video 1. (b) Angiography assessment of graft patency. Arrows indicate the graft location. Scale bar, 1 cm. (c) SEM of day-90 explant. Vessel split longitudinally; scale bar, 1 mm. Higher magnification micrographs (right) show luminal surface at the anastomosis (suture indicated by the arrowhead; scale bar, 50  $\mu$ m) and mid-graft (scale bar, 5  $\mu$ m). (d) Coverage of lumen by endothelial cells (vWF, red). Nuclei counterstained by DAPI (blue); scale bar, 500  $\mu$ m. (e) Distribution of endothelial cells (vWF, red) and smooth muscle cells ( $\alpha$ -SMA, green); scale bar, 100  $\mu$ m. (f) Transmission electron microscopy of interface between endothelial cells (EC) and smooth muscle cells (SMC). Arrowheads denote a basement membrane. Scale bar, 2  $\mu$ m.



Graft material is crucial for host remodeling. When we substituted PGS with PCL, with all other graft parameters being identical, graft remodeling was substantially impaired (Supplementary Fig. 5). Only a thin layer of smooth muscle was observed near the lumen. This is consistent with results of other cell-free approaches in arterial tissue engineering where a thin layer of endothelial cells and small amount of smooth muscle were present with graft materials largely intact. Cells within the interstitial space of the graft wall were negative for  $\alpha$ -SMA and were potentially inflammatory cells or fibroblasts (Supplementary Fig. 4b). PCL grafts showed poor integration with host tissue even at 90 d, at which a clear boundary was visible and the grafted segment distorted the aorta (Supplementary Fig. 6). Collagen I expression was much higher than collagen III and elastin expression. Collagen I distribution was extensive, spanning the whole graft area from lumen to albumen (outer surface), whereas other ECM proteins were mostly expressed only near the lumen (Supplementary Fig. 6c–f). These results, coupled with the 'walled-off' appearance of the H&E stained graft, suggest that collagen I might serve to isolate the PCL from the host.

## DISCUSSION

Cell-free approaches to tissue engineering are still relatively new and scarce, with a focus on slow-degrading polymers. A 1-mm polylactide graft was successfully endothelialized in rats, and 10-mm composite polylactide-polyglycolide grafts showed good patency in canine and porcine models<sup>7,23,24</sup>. However, the grafts degraded slowly, limiting cell penetration and causing prolonged presence of foreign materials. Additionally, no compliance data or ECM content were reported. Porcine small intestinal submucosa grafts showed variable patency above 3-mm diameter<sup>25–28</sup>, but smaller grafts failed by acute thrombosis, despite heparin soaking and systemic heparinization<sup>29</sup>. A 4-mm graft based on small intestinal submucosa was populated by endothelial and smooth muscle cells by 3 months in rabbits<sup>30</sup>. However, no mechanical data or ECM content were reported, and the bradykinin vasorelaxation was counterphysiologic. Poly(ester urethane)urea elastomeric grafts were recently shown to be populated by endothelial and smooth muscle cells<sup>31</sup>. Grafts contained both collagen and elastin, but the graft material appeared largely intact at 6 months. A PCL and hydroxyapatite scaffold infused with growth factors regenerated the articular surface of rabbit cartilage<sup>32</sup>; however, resorption of hydroxyapatite is very slow.

Our philosophy departs from the above in that we emphasize rapid graft degradation and host remodeling. To the best of our knowledge, the use of fast-degrading synthetic grafts is a new design perspective to vascular substitutes. This report is a first step that shows promising results, with a nearly complete host remodeling and good integration within 3 months. We believe three features of the reported graft are important: fast degradation, mechanical properties and pore size. We suspect that rapid graft degradation is the most crucial, because it probably induces different inflammatory responses from long-lasting materials. Rapid degradation progressively generates more space for cell infiltration, proliferation and matrix production. Furthermore, fast degradation reduces the duration of host exposure to foreign material. Long-lasting materials cause tissue stiffening from fibrous encapsulation and can activate inflammatory cells to induce neointimal hyperplasia<sup>33</sup>. Matching mechanical properties and optimizing graft pore size may further promote host remodeling and integration. Matching arterial mechanical properties is likely to promote vascular cell differentiation and avoid stress shielding<sup>34</sup>. PGS is the primary component of our grafts, and its modulus is

close to that of native aortas ( $148 \pm 55$  kPa versus  $390 \pm 191$  kPa) at the strains experienced by new grafts over 80–120 mm Hg. Small pores in our grafts probably pack infiltrating cells close together to promote self-assembly<sup>13</sup>.

As with any new technology, many questions remain to be answered. The applicability of this approach in large animals awaits investigation. Rats have different regeneration potential, and their endothelial cells have a greater capacity to cover the lumen and the grafts than those of humans (especially aged humans)<sup>35,36</sup>. It is crucial to investigate the rate of graft endothelialization and the safety of surgeries with no or low heparin in large animals. The rats we used were healthy, because there are few atherosclerotic animal models with suitable artery sizes. Up to now, only apolipoprotein E-deficient mice are available and widely accepted.

The type and quantity of cells recruited to the graft and their changes over time remain to be studied. The vWF- and  $\alpha$ -SMA-negative cells in the neoartery wall at 14 d may be a mixture of progenitor and inflammatory cells. Macrophages are crucial in vascular graft remodeling<sup>8,37</sup>. A substantial fraction of infiltrating macrophages in our grafts expressed the M2 macrophage marker CD163. A previous study found that mouse vena cava grafts with higher M2 macrophage activation stenosed more<sup>37</sup>. In contrast, none of our grafts showed stenosis, even at 90 d. This could be due to the difference in graft material and design or differences in the animal models. A large number of smooth muscle cells are present in the neoartery wall. Tracing their origin may reveal how to accelerate their recruitment. Progenitor cells in the adventitia of the vena cava and the fascia could facilitate regeneration via paracrine effects or contribute directly to the regeneration of the neoartery. Consequently, parts of grafts contacting the cava and fascia might form a thicker wall more rapidly, accounting for the nonuniform thickness of neoarteries (Fig. 3a).

In addition to increasing antithrombogenicity, heparin is known to bind, stabilize and potentiate the activity of a wide variety of bioactive molecules<sup>38</sup>. Heparin could localize fibroblast growth factor and vascular endothelial growth factor families of angiogenic factors to grafts, promoting vascular cell infiltration. Heparin may also promote cellular infiltration by binding cell adhesion proteins such as PECAM-1 and L-selectin, adhesive matrix proteins and chemokines<sup>38</sup>. Previous work demonstrated that heparinization improves implant cellularity and angiogenesis<sup>39</sup>. Heparin can also promote remodeling by binding remodeling factors such as tissue plasminogen activator<sup>38</sup>.

PCL improves graft sutureability; however, it might not be the optimal sheath material. Residues of graft materials observed at 3 months are probably PCL, because solid nonporous PGS degrades within 2 months subcutaneously<sup>40</sup>. The presence of graft material when the neoartery is sufficiently strong and compliant is likely to inhibit host remodeling. Thus, investigation of sheath materials with mechanical properties similar to PCL but with a faster degradation is warranted. This research may lead to advanced biomaterials and graft designs that can bring synthetic small arterial grafts closer to clinical translation. Furthermore, the focus on rapid graft remodeling is probably applicable to tissues other than blood vessels, especially when combined with progenitor-cell homing signals.

## METHODS

Methods and any associated references are available in the online version of the paper.

*Note: Supplementary information is available in the online version of the paper.*

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## AUTHOR CONTRIBUTIONS

W.W. designed experiments, fabricated and implanted the grafts, characterized explants and analyzed data. R.A.A. performed mechanical characterization of grafts and explants, and analyzed data. Y.W. designed experiments and supervised the project. All authors interpreted results and contributed to writing the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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